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**Abstract:** Three crude polysaccharides of RCP-A, B, and C were derived from the rhizome of *Rohdea chinensis* by means of hot water extraction, gradient ethanol precipitation and dialysis. Three different polysaccharides of RCP-C1-1, RCP-C1-2, and RCP-C1-3 were isolated using cellulose DEAE-52 and Sephadex G-200 chromatography from RCP-C. The average molecular weights of RCP-C1-1, RCP-C1-2, and RCP-C1-3 were measured as  $1.51 \times 10^4$ ,  $1.06 \times 10^4$ , and  $4.86 \times 10^3$  by means of MALDI-TOF MS and UHPGC, respectively. All three polysaccharides were found to consist of D-fructose and D-glucose following hydrolysis and comparison with literature data. Based on FT-IR and NMR analysis, the polysaccharides were identified as inulin-type fructans, with their backbone composed of  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-( $\beta$ -D-fructofuranosyl) $_n$ -(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside ( $n_{RCP-C1-1}=79$ ,  $n_{RCP-C1-2}=58$ ,  $n_{RCP-C1-3}=27$ ). The anti-tumor activity of the polysaccharides (RCP-A, B, and C) was evaluated in H22 tumor-bearing mice. The results suggested that the polysaccharides (RCP-A, RCP-B, and RCP-C) inhibited the growth of H22 hepatocellular. Further, the treated groups of RCP-A, RCP-B, and RCP-C exhibited improvements in body weight as well as spleen/thymus indexes in H22 tumor-bearing mice.

**Keywords:** *Rohdea chinensis*; polysaccharides; anti-tumor activity; inulin-type fructans. © 2024 ACG Publications. All rights reserved.

### 1. Introduction

*Rohdea chinensis* was revised by *Tupistra chinensis* and is recognized as a traditional Chinese ethnic herb, exhibiting properties akin to both medicine and food [1]. Its rhizomes are primarily used to treat sore throat, stomachache, and bruised injuries [2]. Compounds and their derivatives from

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## Polysaccharides from *Rohdea chinensis*

*Rohdea chinensis* have been extensively adopted in the food, medicine, and cosmetics industries [3]. Researchers have identified that the pharmacological effects of *Rohdea chinensis* include cytotoxic, anti-inflammatory, anti-bacterial, and anti-tumor activities [4,5], and the chemical constituents mainly include steroids, flavonoids, alkaloids, volatile oils, polysaccharides [6]. In contemporary times, polysaccharides are extensively employed in various sectors such as healthcare, functional food production, cosmetics, pharmaceuticals, and other manufacturing industries [7]. Polysaccharides exhibit significant anti-tumor effects with minimal side effects, rendering them a focal point in ongoing anticancer drug research and offering considerable potential for widespread application [8]. Hepatocellular carcinoma (HCC) constitutes 90% of all primary malignant liver tumors, posing a significant health concern [9]. Further, there is abundant evidence supporting the idea that the anti-tumor activities of various chemotherapeutic agents result in toxicity to normal cells and damage to organs [10]. In previous research, it was indicated that crude polysaccharides from *Rohdea chinensis* exhibited significant inhibitory activity in H22 tumor-bearing mice [11]. As such, the objective of the present study was to investigate the active polysaccharides of *Rohdea chinensis* with the goal of enhancing immunity and inhibiting cancer cell proliferation. This endeavor could contribute to furthering the pharmaceutical potential of *Rohdea chinensis*.

## 2. Materials and Methods

### 2.1. Materials

Dextran T1000, Dextran T2000, Dextran T6000, Dextran T10000, Dextran T20000 were purchased from Macklin (Shanghai, China). DEAE-52 and Sephadex G-200 were purchased from Kermel (Tianjin, China). Analytical chemicals such as ethanol were purchased from Fuyu Chemical (Tianjin, China). 5-fluorouracil injections were purchased from Xudong HaipuPharmaceutical Co., Ltd (Shanghai, China). 4-6 weeks old BALB/c mice were provided by the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China).

### 2.2. Plant Material

The herbal species was gathered from Changyang Tujia Autonomous County, Yichang City, Hubei Province, China, and was identified by Professor Chen Faju of the College of Biological and Pharmaceutical Sciences of China Three Gorges University as *Rohdea chinensis*, and a voucher specimen (2019052402) was deposited in the herbarium.

### 2.2. Extraction and Ethanol Precipitation

Rhizomes of *Rohdea chinensis* (50.0 kg) were washed and chopped, and then degreased with 95% ethanol for three times at 65°C. The remaining residue underwent hot water extraction for three times to obtain crude polysaccharides [12]. These polysaccharides were precipitated using varying volumes of ethanol to achieve final concentrations of 30% and 50%, resulting in the formation of RCP-A, RCP-B, and RCP-C after dialysis and depigmentation [13,14].

### 2.3. Isolation and Purification

Among RCP-A, RCP-B, and RCP-C, after protein removal via the Sevage method [15], RCP-C was subjected to cellulose DEAE-52 chromatography column separation, with elution using H<sub>2</sub>O, 0.1 M NaCl, 0.3 M NaCl, and 0.7 M NaCl (RCP-C1, RCP-C2, RCP-C3, and RCP-C4) [16]. Subsequently, RCP-C1 was subjected to Sephadex G-200 chromatography column, eluting with water, resulting in the isolation of RCP-C1-1, RCP-C1-2, and RCP-C1-3. Filtrate collection was performed at 10 mL intervals, and each filtrate was analyzed using the phenol-sulfuric acid method.

#### 2.4. Analysis of Monosaccharide

For the hydrolysis and determination of polysaccharides, reference was made to the method of De Ruiter *et al.* with minor modifications [17]. In brief, RCP-C1-1 (5 mg) was hydrolyzed in chromatography vial with trifluoroacetic acid solution (1 mL, 2 M) at 60°C for 1 h.

After washing with methanol for 3 times and drying, the samples were dissolved in water for detection purposes. Additionally, a set of monosaccharide standards were prepared for comparison. The aforementioned samples were tested using the Thermo IC S5000 plus ion chromatography system connected with the Dionex™ CarboPac™ PA20 liquid chromatography column (150×3.0 mm, 10 μm). The mobile phases A, B, and C were H<sub>2</sub>O, 0.1 M NaOH, and 0.1 M NaOH/0.2 M NaAc, respectively.

#### 2.5. Determination of Molecular Weight of the Fractions

The molecular weights of RCP-C1-1, RCP-C1-2, and RCP-C1-3 were determined using Bruker Daltonics MALDI-TOF MS. Additionally, an alternative method employing ultra-high performance gel chromatography (UHPGC), as described by Zhou *et al.* with minor adjustments, was also employed to assess their average molecular weight [18]. Briefly, the three polysaccharides and Dextrans were analyzed using the Dionex Ultimate 3000 UPLC system coupled with the Shodex RI-101 refractive index detector and TOSOH TSK-GEL G4000 PWXL gel column. The mobile phase consisted of H<sub>2</sub>O.

#### 2.6. The Analysis of FT-IR and NMR

The samples were prepared by mixing 5 mg of polysaccharides (RCP-C1-1, RCP-C1-2, and RCP-C1-3) and 250 mg of KBr. The absorption spectra were recorded on a Frontier IR. Structure information of the homogeneity was obtained by means of FT-IR spectroscopy. The three dried polysaccharides (200 mg each) were dissolved in a mixed solvent comprising 500 μL D<sub>2</sub>O and 40 μL C<sub>3</sub>D<sub>6</sub>O. Subsequently, they were subjected to analysis using a Bruker 400 MHz NMR spectrometer.

#### 2.7. Animal Experimentation and Administration Method

For the H22 model and administration method of hepatocellular carcinoma mice, reference was made to Yang *et al.*'s method with minor modifications [19]. The ascites cells of BALB/c mice aged 4-6 weeks were diluted with normal saline to 1×10<sup>7</sup> cells/mL injected into the right axillary area of each mouse (0.2 mL). The mice were randomly divided into the following groups: model group, 5-fluorouracil (5-FU) group, and RCP-A, RCP-B, and RCP-C groups, each administered with varying dosages (40, 20, and 10 mg/kg/d). Mice in the model group were given normal saline daily. The 5-FU group was gavaged with 5-fluorouracil (20 mg/kg/d). The high, medium, and low dose groups were treated with polysaccharides (40, 20, and 10 mg/kg/d), respectively. All the administrations were conducted continuously for 15 consecutive days on all BALB/c mice.

### 3. Results and Discussion

#### 3.1. Isolation and Purification

RCP-C1-1, RCP-C1-2, and RCP-C1-3 were obtained from RCP-C after elution using the DEAE-52/Sephadex G-200 chromatography column (Figure S1). The three polysaccharides were finally detected by means of UHPGC as a single peak, indicating their homogeneous nature (Figure S2). However, achieving homogeneous polysaccharides from RCP-A/B proved challenging in the current study due to their poor water solubility. Further research will be conducted in the future to address this issue.

### 3.2. Structure Characterization

The average molecular weights of RCP-C1-1, RCP-C1-2, and RCP-C1-3 were determined using MALDI-TOF MS to be  $1.45 \times 10^4$ ,  $1.06 \times 10^4$ , and  $4.96 \times 10^3$ , respectively, as illustrated in Figure S3. Further, utilizing the retention times of various Dextrans to establish a standard curve, the equation was determined to be  $y = -8298.7x + 168760$ ,  $R^2 = 0.99$ . Based on the retention times (Figure S2) of RCP-C1-1 (18.500 min), RCP-C1-2 (19.132 min), and RCP-C1-3 (19.765 min), the average molecular weights were calculated as  $1.52 \times 10^4$ ,  $9.99 \times 10^3$ , and  $4.74 \times 10^3$ , respectively. These findings demonstrate consistency between the results obtained from the two methods.

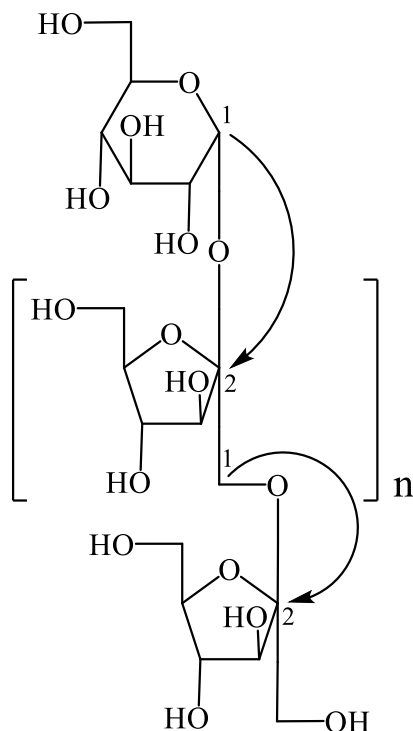
The FT-IR results, depicted in Figure S4, reveal distinctive absorption peaks. The absorption peaks within  $1150\text{--}1000\text{ cm}^{-1}$  were indicative of pyran glycosidic bonds [20]. Moreover, the absorption peaks at  $1000\text{--}800\text{ cm}^{-1}$  suggested both  $\alpha$  and  $\beta$ -configurations [21-23]. The ion chromatograms of standard monosaccharides and RCP-C1-1 are illustrated in Figure S5, indicating that the monosaccharide composition in RCP-C1-1 consists of glucose and fructose with a molar ratio of 1.23:98.59.

The  $^1\text{H}$ -NMR spectra (Figure S6) of the three polysaccharides exhibited analogous proton signals at  $\delta_{\text{H}}$  3.00-6.00 ppm. The proton signals at  $\delta_{\text{H}}$  5.00-5.40 ppm were identified as the anomeric protons of the  $\alpha$ -configuration [24]. The only difference observed was in the integration ratio of proton signals at  $\delta_{\text{H}}$  5.30-5.35 ppm (Glu-H1) to those at  $\delta_{\text{H}}$  4.10-4.20 ppm (Fru-H3), which was utilized to determine the degree of polymerization (DP) of the three polysaccharides. Consequently, the DPs of the three polysaccharides were indicated to be 84, 59, and 27, which were consistent with MALDI-TOF MS. In the  $^{13}\text{C}$ -NMR spectra of the three polysaccharides (Figure S7), the three polysaccharides demonstrated similar carbon signals at  $\delta_{\text{C}}$  60.00-104.00 ppm. These signals were verified as three tertiary carbon signals at  $\delta_{\text{C}}$  81.05-81.10 ppm,  $\delta_{\text{C}}$  76.55-76.60 ppm, and  $\delta_{\text{C}}$  74.35-74.50 ppm, along with two secondary carbon signals at  $\delta_{\text{C}}$  61.85-62.10 ppm and  $\delta_{\text{C}}$  60.00-60.50 ppm based on the DEPT 135 spectra (Figure S8). These findings were consistent with the HSQC spectrum (Figure S9) and were supported by the assignment data of proton/carbon signals (Table 1) [25]. In addition, within the anomeric carbon region, the carbon signals at  $\delta_{\text{C}}$  103.30-103.80 ppm could be ascribed to  $\beta$ -D-Fru [26]. In the HMBC spectra (Figure S10), the observed correlations between H1-Fru/C2-Fru and H1-Glu/C2-Fru indicated the presence of (1 $\rightarrow$ 2)-linked  $\beta$ -D-fructofuranosyl and terminal glucose [27,28].

As fructose polymers, inulin-type fructans can be characterized by predominantly or exclusively (1 $\rightarrow$ 2)- $\beta$ -D-fructosyl- $\beta$ -D-fructose linkages, mostly linear molecules, and can typically terminate with a singular glucose unit through an  $\alpha$ -D-glucopyranosyl bond [29]. Based on the provided data, the structure of the three polysaccharides were inferred to be  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-( $\beta$ -D-fructofuranosyl) $n$ -(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside ( $n_{\text{RCP-C1-1}}=79, n_{\text{RCP-C1-2}}=58, n_{\text{RCP-C1-3}}=25$ ), as shown in Figure 1.

**Table 1.**  $^1\text{H}/^{13}\text{C}$  NMR data for RCP-C1-1, 2, and 3

NO. of H/C	RCP-C1-1		RCP-C1-2		RCP-C1-3		Inulin-type fructan	
	$^1\text{H}$ NMR	$^{13}\text{C}$ NMR	$^1\text{H}$ NMR	$^{13}\text{C}$ NMR	$^1\text{H}$ NMR	$^{13}\text{C}$ NMR	$^1\text{H}$ NMR	$^{13}\text{C}$ NMR
1	3.84, 3.55	60.53	3.85, 3.59	60.15	3.85, 3.59	60.12	3.81, 3.59	60.79
2	-	103.73	-	103.29	-	103.36	-	103.15
3	4.14	76.70	4.17	76.58	4.16	76.67	4.16	76.88
4	4.00	74.82	4.02	74.46	4.03	74.60	4.00	74.16
5	3.84	80.77	3.78	80.59	3.78	80.72	3.76	80.98
6	3.70, 3.64	62.96	3.72, 3.67	62.31	3.72, 3.66	62.27	3.71, 3.66	62.04



**Figure 1.** Structure of RCP-C1-1, 2 and 3

### 3.3. Activities of Polysaccharides (RCP-A, B, and C) on General Health Status and Body Weight of H22 Tumor-Bearing Mice

The model group exhibited significantly reduced food and water intake, along with a notable decrease in activity frequency. Additionally, these mice displayed unkempt fur and a lack of luster in their appearance. Conversely, mice in the polysaccharides-treated groups demonstrated robustness, characterized by lustrous fur and notable increases in body weight, indicative of a favorable overall condition. Moreover, toxicity observed in the 5-FU group was notably severe, manifesting as significant weight loss, deteriorated fur color, and delayed activity, ultimately leading to a poor physical condition (Table 2).

The body weights of mice in the polysaccharides-treated group exceeded those of the model group. In contrast, compared to the model group, the body weight increase in the 5-FU group was significantly reduced ( $P < 0.05$ ).

### 3.4. Activities of Polysaccharides (RCP-A, B, and C) on Tumor Growth and the Immune Organ Index of H22 Tumor-Bearing Mice

Numerous studies have shown that tumor suppression is assessed by the reduction of tumor masses, therefore, tumor inhibitory rate often serves as an indicator to assess anti-tumor agents [30]. In the present study, the anti-tumor activities of polysaccharides were evaluated in H22 tumor-bearing mice at doses of 10, 20, and 40 mg/kg. From the data presented in Table 2, it was observed that the tumor weight in the polysaccharides-treated groups at all three doses, as well as the 5-FU group, showed a significant reduction compared to the model group ( $P < 0.05$ ). The polysaccharide groups achieved a maximum inhibition rate of 52.37%. Overall, for the spleen/thymus index, there was no significant difference between polysaccharides-treated groups and the tumor control group. However, a significant distinction was observed between the 5-FU group and the polysaccharide groups ( $P < 0.05$ ). Based on the described data, it can be concluded that the polysaccharides-treated groups demonstrated significant activity in inhibiting tumor growth and increasing the mass of the spleen/thymus in H22 tumor-bearing mice.

**Table 2.** Effect of RCP-A, B and C on Tumor Growth and the Immune Organ Index of H22 Tumor-bearing Mice<sup>a</sup>.

Group	Dosage (mg/kg/d)	Body weight change (g)	Tumor weight (g)	Inhibition rate (%)	Thymus index (mg/g)	Spleen index (mg/g)
Model	-	4.33±3.83 <sup>##</sup>	1.25±0.24 <sup>##</sup>	-	1.87±0.82	7.83±2.75
5-FU	20	0.11±5.43	0.28±0.09	77.08	0.44±0.32	5.52±1.73
	40	5.34±3.71 <sup>##</sup>	0.83±1.27 <sup>*</sup>	33.88	1.88±1.48 <sup>##</sup>	7.84±0.58 <sup>#</sup>
RCP-A	20	5.63±4.32 <sup>##</sup>	0.92±0.28 <sup>*</sup>	26.51	1.49±0.66 <sup>##</sup>	8.65±2.15 <sup>#</sup>
	10	6.03±6.87 <sup>##</sup>	0.85±0.58 <sup>*</sup>	32.01	2.66±0.65 <sup>*##</sup>	8.26±0.50 <sup>#</sup>
	40	5.72±4.93 <sup>##</sup>	0.99±0.37 <sup>**</sup>	28.59	1.44±0.89 <sup>##</sup>	6.68±1.02 <sup>#</sup>
RCP-B	20	2.21±4.06 <sup>##</sup>	0.59±0.07 <sup>**</sup>	52.37	2.35±0.62 <sup>*##</sup>	8.81±1.65 <sup>#</sup>
	10	3.02±3.26 <sup>##</sup>	1.01±0.24	19.60	2.68±0.92 <sup>*##</sup>	8.73±0.79 <sup>#</sup>
	40	5.02±4.47 <sup>##</sup>	0.62±0.29 <sup>**</sup>	50.25	1.35±0.95 <sup>##</sup>	7.77±0.71 <sup>#</sup>
RCP-C	20	3.62±3.96 <sup>##</sup>	0.72±0.64 <sup>*</sup>	42.50	1.31±1.14 <sup>##</sup>	7.54±1.21 <sup>#</sup>
	10	3.22±4.35 <sup>##</sup>	0.63±0.34 <sup>**</sup>	49.96	0.91±0.79 <sup>#</sup>	7.95±2.40 <sup>#</sup>

<sup>a</sup> Values are expressed as means ± S.D. (n=10).

\* P < 0.05 as compared with the model group.

\*\* P < 0.01 as compared with the model group.

# P < 0.05 as compared with the 5-FU group.

## P < 0.01 as compared with the 5-FU group.

#### 4. Conclusion

The RCP-C1-1, RCP-C1-2, and RCP-C1-3 obtained from *Rohdea chinensis* were identified as amorphous powder, comprising two monosaccharide molecules: D-fructose and D-glucose. Analysis using UHPLC and MALDI-TOF MS indicated that the average molecular weights of RCP-C1-1, RCP-C1-2, and RCP-C1-3 were  $1.51 \times 10^4$ ,  $1.06 \times 10^4$ , and  $4.86 \times 10^3$ , respectively. Based on this data and structural analysis, it can be reasonably inferred that the backbone of the three polysaccharides was composed of (1→2)-linked- $\alpha$ -D-Glc and (1→2)-linked- $\beta$ -D-Fru, with an approximate molecule structure of  $\alpha$ -D-Glu-(1→2)-( $\beta$ -D-Fru)<sub>n</sub>-(1→2)- $\beta$ -D-Fru ( $n_{\text{RCP-C1-1}}=79$ ,  $n_{\text{RCP-C1-2}}=58$ ,  $n_{\text{RCP-C1-3}}=27$ ). The polysaccharides (RCP-A, B, and C) demonstrated anti-tumor activity in H22 tumor-bearing mice, showing efficacy in inhibiting tumor progression *in vivo* while also increasing body weight, spleen index, and thymus index. Such findings expand the potential applications of *Rohdea chinensis* as both a therapeutic agent and functional food.

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#### Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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Polysaccharides from *Rohdea chinensis*

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