Separation of the Polysaccharides in *Caulerpa racemosa* and Their Chemical Composition and Antitumor Activity

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ABSTRACT: *Caulerpa racemosa* was extracted with the combined procedure of neutral protease and boiling water to yield a water-soluble polysaccharide coded as CRP. The obtained *C. racemosa* polysaccharide (CRP) was fractionated with DEAE-52 cellulose, which led to two soluble polysaccharide fractions designated as CRPF1 and CRPF2. CRP, CRPF1, and CRPF2 were chemically determined and their antitumor activity was assayed. Results showed that they were all mixtures composed of sulfated polysaccharide with 3.9–7.9% uronic acid and protein, and had similar amino acid composition, but CRP was more than CRPF1 and CRPF2 in total sugar and uronic acid content, and less in sulfate group content. Two main sugars, glucose (Glu, 56.8%) and galactose (Gal, 31.8%), of CRP were enriched to CRPF2 (Glu, 89.1%) and CRPF1 (Gal, 99.2%) respectively; Bioassay indicated that all CRP had strong antitumor activity in both *in vitro* and *in vivo*, and its inhibition rate of K562 cells *in vitro* at the concentration of 6.0–10.0 mg mL^{-1} and of H22 tumor transplanted in mice at a dose of 100 mg kg^{-1} day were 59.5–83.8% (48 h) and 53.9% (14 days), respectively. Moreover, at a lower dose (0.05–0.2 mg mL^{-1}) and longer time (72 h), CRP exhibited stronger inhibition effect on K562 cells than CRPF1 and CRPF2. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 1435–1440, 2008

Key words: *Caulerpa racemosa*; polysaccharides; separation; chemical composition; antitumor activity

INTRODUCTION

In recent years, medical potential of seaweed sulfated polysaccharides has drawn more and more attention to scientists. Antitumor, antivirus, antihyperlipidemia, and anticoagulant biological activities have been found in seaweed polysaccharides, some of which have been developed as new drugs.[1–3] However, algal-active polysaccharides have been studied mainly from red seaweeds and brown seaweeds, and fewer from green algae.

Caulerpa racemosa, a large edible green alga, is widely distributed in tropical and subtropical areas, such as the coasts of Guangdong and Taiwan provinces in China and Pacific east coasts.[4] Ji and Zhao analyzed[5] the chemical constituents of *C. racemosa*, Mackie and Percival[6–8] reported the presence of water-soluble polysaccharides in *Caulerpa filiformis*, whereas Rao[9] studied the structural features of a sulfated polysaccharide of *Caulerpa taxifolia*, Cavas et al.[10] investigated the antiproliferative and apoptotic effects of both *C. racemosa* var. cylindracea extract and purified CPN on SHSY5Y and Kelly cell lines, Ghosh et al.[11] studied the antiviral activity of sulfated polysaccharide fractions from *C. racemosa*. However, no studies on the antitumor activitiy of the polysaccharides present in *C. racemosa* have, to the best of our knowledge, yet been reported.

The purpose of the present study was to isolate polysaccharide fractions from the green alga *C. racemosa*, determine their chemical composition, and to investigate their antitumor activity against K562 and H22 cell lines.

EXPERIMENTAL

Materials

Caulerpa racemosa was collected in South China Sea, Zhanjiang, Guangdong province (China) in April 2004. The fresh algae collected was repeatedly washed with fresh water to remove sands and impurity, then dried to 12% water content in the sun, and then completely dried in the oven of 50°C, ground to powder, passed through 60-mesh, and stored in plastic bags at room temperature in a dry and dark place before use.

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All Kunming little white mice for experiment, weighted 20 ± 2.0 g, were purchased from Guangdong Medical College, Zhanjiang city, Guangdong province, People's Republic of China.

DEAE-52 cellulose for polysaccharides separation and sepharose 4B for polysaccharides molecular weight estimation were of Whatman Co. (England), and Dingguo Biotec. Co. respectively; glucuronic acid and glucan standard used were all purchased from Sigma Chemical Co., USA; All reagents utilized were analytical grade bought from local Chemical Reagent Corp. (Guangzhou, China) Phosphate buffer solution (PBS) was prepared by dissolving 8.812 g NaCl, 0.201 g KCl, 1.150 g Na_2HPO_4, and 0.204 g KH_2PO_4 in 1 L of ultrapure water.

Extraction and separation

Four hundred grams of dry alga powder was treated with neutral proteinase for 2.5 h at 60°C (pH 6.5) in 12-fold volume of water, then extracted for 2.5 h at 100°C (pH 6.5) in 20-fold volume of water, and afterwards filtrated through cotton gauze. Filtrate obtained was condensed to 1/4 of its original volume by using rotating vacuum evaporator, then centrifuged at 6000 rpm for 30 min. The supernatant was repeatedly treated with sevage reagent (a mixture of butyl alcohol and chloroform, 1 : 3, v/v) to remove free protein, then precipitated with the addition of threefold volume of 95% (v/v) ethanol and lyophilized to yield 28 g *C. racemosa* polysaccharide (CRP); CRP was fractionated with DEAE-52 cellulose anion exchange column as stationary phase, and with 0.1, 0.5, 1.0, and 2.0 mol L^{-1} NaCl as mobile phases. Fractions eluted with 0.5 and 1.0 mol L^{-1} NaCl were freeze-dried, respectively, and designated as CRPF1 and CRPF2, respectively.

Analysis of chemical composition

Protein content was measured by the method of Bradford[12] using bovine serum albumin as a standard. Uronic acid content was measured by the carbazole reaction,[13] using glucuronic acid as a standard. A sample of 0.5 mL was carefully added to a test tube of 3 mL H_2SO_4 (sp. gr. 1.84). The contents of the tube were then agitated and cooled with flow water to ensure that the temperature of the test solution did not rise above 20°C. When thoroughly mixed, the solution was heated and maintained at 100°C for 10 min, then cooled to 20°C prior to the addition of 0.1 mL of carbazole reagent (0.125% carbazole in ethanol). The mixture was reheated, kept at 100°C for 15 min, and cooled. Absorbance was measured at 540 nm against a reagent blank. The uronic acid content was read from D-glucuronic acid standard curve. Carbohydrate content was measured by the phenol–sulfuric acid method.[14] In brief, 1.0 mL of sample solution was vortex-mixed with 1.0 mL of 5% phenol in water before adding 5 mL of concentrated sulfuric acid rapidly from a glass dispenser. After standing for 30 min at room temperature, the absorbance of the sample solution was measured at 490 nm against the blank (prepared by substituting distilled water for the sample solution). The amount of total carbohydrates was determined by reference to a standard curve made from glucose and galactose. Sulfate group content was detected by the turbidity method using sulfuric acid.[15] A sample of 1.5 mg was added to a test tube of 1.0 mL of 1.0 mol L^{-1} HCl, sealed, maintained at 100°C for 6 h, then cooled, and centrifugalized. A supernatant of 0.2 mL was added to a test tube, 0.38 mL of 3% trichloroacetic acid solution and 1.0 mL glutin solution of $BaCl_2$ were added, respectively, and mixed. Mixtures were stayed for 15–20 min, and their turbidities were determined at 360 nm. Sulfate group content was read from a standard curve made by using K_2SO_4.

Sugar composition of CRP and CRPF was determined by gas chromatography (GC) method, in which the alditol acetate derivatives of the neutral sugars were measured.[16] The GC conditions used was injector temperature, 200°C; column temperature, 220°C; detector temperature, 220°C; carrier gas, N_2; and flow rate, 23 mL min^{-1}. Under these conditions, a GC (GC-17A, from Shimadzu Corp., Japan) fitted with a flame-ionization detector and Shimadzu capillary column DB-1 (Φ 0.25 mm × 30 m, carrier: 100% dimethyl polysiloxane) at 220°C allowed accurate determination of the individual sugars in the standard sugar mixture and internal standard (allose) used for calibration and determination of the detector response factor of individual sugars. Samples were hydrolyzed by using 6.0 mol L^{-1} HCl and 5.0 mol L^{-1} NaOH, respectively, and amino acid composition was determined with 835-50 amino acid analyzer (from Shimadzu Corp.).

Molecular weight estimation

Molecular weight distribution of CRPF1 and CRPF2 was estimated by gel permeation chromatography. Sepharose 4B was packed in a column of 1.6 × 80 cm dimension. The flow rate of the packed column was adjusted to 1 mL min^{-1} with a peristaltic pump. Sample of 0.5 mL was added to the column. The eluants from the chromatographic system were collected by a fraction collector (3.0 mL eluant/tube). The bed volume of the column was determined by using Blue glucan (MW: 2.0×10^6 Da, 4.0 mg mL^{-1}). To describe the standard curve between logarithm of polysaccharides molecular weight and elution volume, a series of standard molecular weight glucans

such as T-70 (6.85×10^4 Da), T-110 (1.1×10^5 Da), T-500 (4.5×10^5 Da), and T-2000 (2.0×10^6 Da) were added to the column and eluted respectively, and the elution peaks of standard glucans were detected by measuring optical density (OD) at 490 nm. Molecular weight distribution of polysaccharide fractions were estimated by measuring OD of the elution peaks of the fractions at 490 nm and referring to a standard curve made from glucans.

Antitumor assay *in vitro*

K562 cells, donated by the Institute of Ocean Medicine of Guangdong Ocean University, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. K562 cell collected from logarithm growing stage was added to a 96-well plate, each containing 90 μL cell suspension (1.0×10^4 cells/well). After cultivation for 24 h in a 5% CO_2 incubator at 37°C, 10 μL of polysaccharides sample at the concentration of 6.0, 8.0, 10.0, 12.0 mg mL^{-1} (or 0.05, 0.1, 0.2 mg mL^{-1}) and the RPMI 1640 of equal volume were added to 96-well plates of experiment group and control group, respectively, and shook completely. After continually cultivating for 24 or 48 h (or 72 h), 20 μL of 3-(4,5-dimethythiazal)2,5-diphenylterazoliumbromide (5 g L^{-1} PBS) was added to each well respectively. After continually incubating for 5 h again, 100 μL reagent consisting of 1.0% sodium dodecyl sulfate, 5.0% isobutyl alcohol, and 0.012 mol L^{-1} HCl was added to each well and maintained overnight at 37°C, and then the OD of each well at 570 nm was determined with the enzyme-marking instrument.[17]

Antitumor assay *in vivo*

Antitumor effect of polysaccharides from *C. racemosa* on H22 cells was examined by inoculating 0.2 mL of PBS containing 2×10^7 tumor cells/mL, which had proliferated for 6 days, subcutaneously, into the left groin of 8-week-old Kunming little white mice (0.2 mL/mouse). The mice were randomly classified into groups (10 mice/group) next day. A solution of test sample was given to mice for 14 days by routine oral passage starting from 24 h after tumor inoculation, once daily (0.5 mL/mouse). Equal volume of PBS was also given to the negative group for 14 days. Mice were weighted 24 h after the last solution of test sample was given to them, and then the solid tumor was excised from mice and weighed. The results of the antitumor *in vivo* were expressed as the inhibition rate of tumor cells calculated as inhibiting rate $= [(C - T)/C] \times 100\%$, where C and T represented the average tumor weight of control group and test group, respectively.[18]

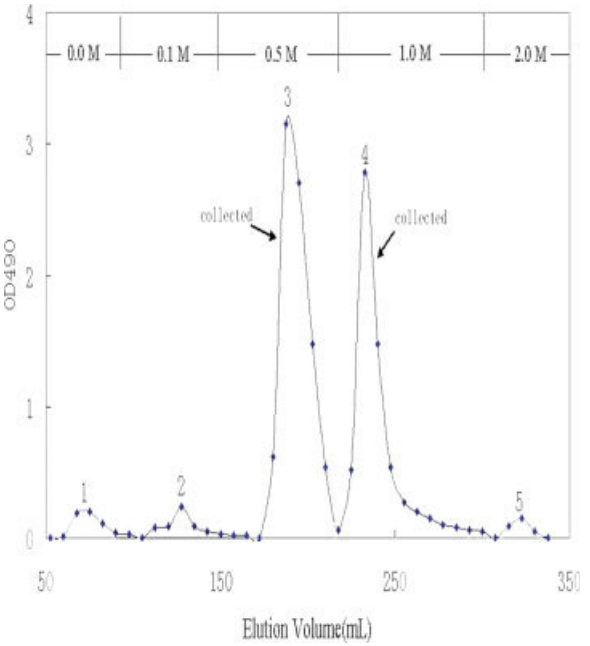

Figure 1 Anion exchange chromatography profile of CRP from *Caulerpa racemosa* on DEAE-52 cellulose with 0.0, 0.1, 0.5, 1.0, and 2.0*M* NaCl, respectively.

Statistical analysis

Data in the bioassays were statistically evaluated by Student's *t*-test, and the differences with a P value of less than 0.05 were considered to be significant.

RESULTS

Extraction and separation

The yield of crude polysaccharide, namely CRP, from *C. racemosa* was between 7 and 8% of the algal dry weight after the first ethanol precipitation, dialysis, and freeze-drying. To obtain purer polysaccharide, CRP was fractionated with DEAE-52 cellulose anion exchange chromatography because they are highly sulfated polysaccharides. Chromatographic profile of CRP with the elution of 0.0, 0.1, 0.5, 1.0, and 2.0 mol L^{-1} NaCl was showed in Figure 1 respectively. It can be seen from Figure 1 that two fractions with the elution of 0.5 and 1.0 mol L^{-1} NaCl accounted for 90% of its total eluants; among them, Fractions 3 and 4 (in Fig. 1) were 47 and 43% of its total eluants, respectively.

Analysis of chemical composition

Chemical compositions of polysaccharides from *C. racemosa* were summarized in Table I. It can be seen from Table I that they were all proteoglycans and rich in sulfate group of 27.6–48.3%. The contents of

TABLE I
Chemical Composition of CRP and CRPFs from *Caulerpa racemosa*

Sample	Total sugar (%)	Uronic acid (%)	Protein (%)	Sulfate group (%)
CRP	53.7	7.9	9.9	27.6
CRPF1	36.0	7.6	14.0	48.3
CRPF2	45.1	3.9	2.0	45.6

their saccharide and protein were 36.0–53.7% and 2.0–14.0%, respectively. Moreover, there were also 3.9–7.9% uronic acid, except neutral sugar.

On analysis of neutral sugar, the predominant sugar of CRP was glucose (Glu, 56.8%), followed by galactose (Gal, 31.8%) and mannose (Man, 11.4%). Two kinds of sugar were only detected in CRPF1 and CRPF2, respectively. Sugar composition of CRPF1 was different from that of CRPF2, and CRPF1 was almost composed of galactose (Gal, 99.2%) except trace amount of glucose (Glu, 0.8%), and the main sugar of CRPF2 was glucose (Glu, 89.1%), followed by mannose (Man, 10.9%).

Amino acid determination of the proteoglycan suggested that amino acid compositions in CRP, CRPF1, and CRPF2 displayed similar change, and their main amino acids all were Asp and Glu, followed by Ala, Glu, Gly, and Val, and very little of Trp and His (Table II). This fact can be related with their bioactivity, because acidic amino acids, especially Asp, play an important role in cell energy and nitrogen metabolism, and in the transferring of nerve substance.

TABLE II
Amino Acid Compositions of CRP and CRPFs from *Caulerpa racemosa* (%)

Category	Symbol	CRP	CRPF1	CRPF2
Acidic amino acid	Asp	2.51	1.53	0.45
	Glu	2.62	1.51	0.51
Neutral amino acid	Thr	0.91	0.58	0.17
	Ser	0.82	0.50	0.15
	Pro	0.73	0.45	0.17
	Gly	1.05	0.67	0.25
	Ala	1.08	0.72	0.29
	Cys	0.16	0.11	0.04
	Val	1.01	0.66	0.27
	Met	0.30	0.20	0.10
	Ile	0.64	0.39	0.16
	Leu	0.82	0.50	0.21
	Tyr	0.35	0.24	0.10
	Phe	0.67	0.37	0.15
	Trp	0.056	<0.05	–
Basic amino acid	Lys	0.84	0.42	0.15
	His	0.08	0.05	0.01
	Arg	0.80	0.44	0.15
Total amino acids		15.40	9.34	3.33

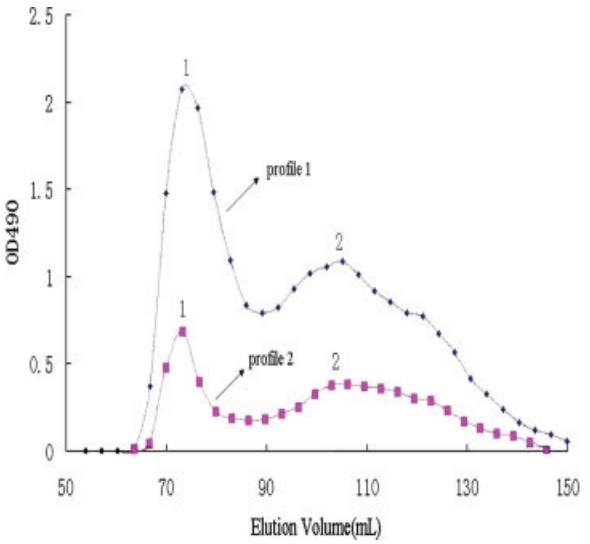

Figure 2 The chromatography profile of CRPF1 and CRPF2 eluted with 0.02*M* NaCl on sepharose 4B column. Conditions: Sample 0.5 mL, 16 mg mL^{-1} of CRPF1, and 4 mg mL^{-1} of CRPF2; Velocity, 1.0 mL min^{-1}. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Molecular weight estimation

To estimate molecular weight distribution of CRPF1 and CRPF2, elution volume of T series of standard molecular weight glucan on sepharose 4B were used to obtain standard regression equation as follows: $V_e = 270.77–32.1861lg\ M_w$, $R^2 = 0.9973$, where V_e represented elution volume, M_w meant molecular weight. Molecular weight distribution of CRPF1 and CRPF2 were estimated according to their elution volume on the sepharose 4B and referring to the regression equation made from glucans. The elution profiles of CRPF1 and CRPF2 on sepharose 4B were showed in Figure 2. CRPF1 and CRPF2 were still a mixture of at least two fractions and had a very similar molecular weight distribution. CRPF1 consisted of 139.5 $\times$ 10^4 Da fraction and 14.4 $\times$ 10^4 Da fraction (Profile 1 in Fig. 2), and CRPF2 was composed of 136.5 $\times$ 10^4 Da Fraction 1 and 9.9 $\times$ 10^4 Da Fraction 2 (Profile 2 in Fig. 2).

Antitumor activity *in vitro*

Evident inhibition effect of CRP on K562 cells was observed in this study (Table III). After 48 h of cultivating K562 cells, the dose–effect relationship of CRP at the concentration of 6.0–10.0 mg mL^{-1} was displayed, but no changed was observed from 10.0 to 12.0 mg mL^{-1}. This indicated that the increment of CRP concentration did not influence its inhibition effect on K562 cells since then.

Polysaccharides of different purity from *C. racemosa* had shown different impact on K562 cells (Fig. 3). The results indicated that CRP, CRPF1, and

TABLE III
Inhibition Effect of CRP on K562 Cell Growth

Group	Concentration (mg mL^{-1})	Time (h)			
		24 h		48 h	
		OD_{570nm}	IR (%)	OD_{570nm}	IR (%)
Control		0.134 ± 0.001	–	0.148 ± 0.000	–
CRP	6	0.081 ± 0.003*	39.6	0.060 ± 0.012**	59.5
	8	0.068 ± 0.006**	49.3	0.033 ± 0.003***	77.7
	10	0.063 ± 0.001**	53.0	0.024 ± 0.003***	83.8
	12	0.065 ± 0.003**	51.5	0.024 ± 0.004***	83.8

IR represents inhibition rate.
* Significant difference ($P < 0.05$) contrasted with control group.
** Highly significant difference ($P < 0.01$).
*** Highly significant difference ($P < 0.001$).

CRPF2 could inhibit the growth of K562 cells at a lower dose (0.05–0.2 mg mL^{-1}) and longer time (72 h). However, CRPF2 activity was significantly lower than that of CRP and CRPF1, and decreased with dose increment. It was conjectured that the optimum dose of CRPF2 might be lower.

Antitumor activity *in vivo*

CRP was assayed for antitumor activity by using H22 tumor in mice (Table IV). Results displayed that CRP of different dose could have significant inhibition on H22 tumor. After 14 days of transplantation, the weight of tumor in mice without CRP increased to 1.02 g. The tumor weight of mice for test group provided with CRP at a dose of 100 mg kg^{-1} days by routine oral passage decreased to 0.47 g, and the inhibition rate of tumor came to 53.9%.

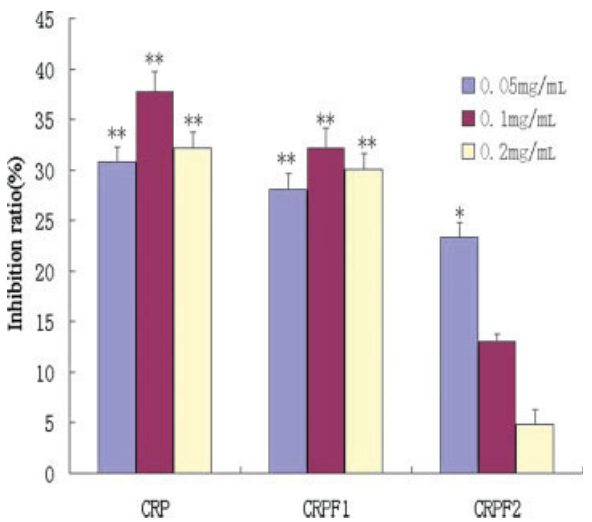

Figure 3 Inhibition effects of CRP, CRPF1, and CRPF2 on K562 cells (72 h). Notes: * means significant difference ($P < 0.05$) contrasted with control group, ** means highly significant difference ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Zhou et al.[19] found that the antitumor and immunomodulation activities of sulfated polysaccharides had positive correlation to sulfate group contents in some scope, namely the richer the sulfated group content was, the higher the antitumor activity of its polysaccharide was. In order not to desulfate during polysaccharide extraction, the combined procedure of neutral protease and boiling water as extracting polysaccharide in *C. racemosa* was adopted, but the yield of CRP obtained from it was only 7–8%. The low yields obtained after such an extraction procedure was likely to be due to loss of CRP during free protein removal, because the presence of sugar in the proteins removed was confirmed by using phenol–sulfate acid method. So more efficient extraction programs need to be developed.

CRPF1 and CRPF2 obtained by eluting CRP on DEAE-52 cellulose anion exchange columns with 0.5 and 1.0 mol L^{-1} NaCl, respectively, were both a mixture of at least two fractions with slight difference in molecular weight distribution, which were

TABLE IV
Inhibition Effect of CRP on H22 Tumor in Mice

Group	Dose (mg kg^{-1} day)	Mice number (n)	Tumor weight (g)	Inhibition rate (%)
Negative control	–	10	1.02 ± 0.35	–
CRP	50	10	0.71 ± 0.30	30.4*
	100	10	0.47 ± 0.19	53.9**
	200	10	0.69 ± 0.21	32.4*

* Significant difference ($P < 0.05$) contrasted with control group.
** Highly significant difference ($P < 0.01$).

confirmed by their elution profile on sepharose 4B column (Fig. 2). The composition analysis of sugar and protein suggested that CRPF1 and CRPF2 were both proteoglycan, but had significant difference in sugar content and chemical composition, sulfation degree, and protein content. This fact could lead to the difference in their antitumor activity.

An indepth knowledge of the proteoglycan composition and chemical structure is a prerequisite to the understanding of their biological and physiochemical properties. In our experiments, the proteoglycans got were still mixtures consisting of at least two fractions, but they all exhibited antitumor activity. To identify their structure and to elucidate the relationship of structure and bioactivity, further purification was still being carried out in our lab.

The results of chemical analysis showed that the polysaccharides from *C. racemosa* contained 3.9–7.9% amount of uronic acid, which was different from the results reported in the literature.[20] Relationship of uronic acid content in the polysaccharide and its activity is not clear, but uronic acid content of polysaccharide in *C. racemosa* and its inhibition effect on K562 cells seemed to be of negative correlation at a dose of 0.05–0.2 mg mL^{-1} provided in this study. However, authenticity of this phenomenon still needs further confirmation.

The results of the bioassays showed that CRP exhibited higher inhibition effect on K562 cells than CRPF1 and CRPF2 (Fig. 3) *in vitro* at a lower dose (0.05–0.2 mg mL^{-1}) and longer time (72 h); in the meantime, negative correlation of CRPF2 activity and dose was also observed, and at a dose of 0.1–0.2 mg mL^{-1} and 72 h, the inhibition rate of CRPF2 against K562 cells was not significantly different when contrasted with the control group ($P < 0.05$). The fact indicated that CRPF2 could have higher inhibition rate on K562 cells *in vitro* at a dose of less than 0.05 mg mL^{-1}. To identify optimum dose of CRPF2 inhibition effect on K562 cells, further study still needs to be undertaken.

CONCLUSIONS

Polysaccharide CRP extracted with the combined procedure of neutral protease and boiling water from *C. racemosa* and CRPF1 and CRPF2 fractionated with DEAE-cellulose 52 from CRP were all mixtures composed of protein and sulfated polysaccharide. They were basically similar in amino acid composition, but had significant difference in sugar composition and sulfate group content. The results of bioassay confirmed that CRP, CRPF1, and CRPF2 had antitumor activity. So polysaccharides in *C. racemosa* can be developed into a potential antitumor drug.

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