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Syntheses of new rare earth complexes with carboxymethylated polysaccharides and evaluation of their in vitro antifungal activities

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ABSTRACT

In the present paper, La, Eu and Yb were selected to represent light, middle and heavy rare earths to form complexes with polysaccharides through chelating coordination of carboxyl groups, which were added into polysaccharide chains by means of carboxymethylation. Their antifungal activities against plant pathogenic fungi were evaluated using growth rate method. These rare earth complexes exhibited various antifungal activities against the tested fungi, depending on rare earth elements, polysaccharide types and fungal species. Among these three metal elements (i.e. La, Eu and Yb), Yb formed the complexes with the most effective antifungal properties. Furthermore, the results showed that ligands of carboxymethylated polysaccharides played a key role in promoting cytotoxicity of the rare earth complexes. Carboxymethylated *Ganoderma applanatum* polysaccharide (CGAP) was found to be the most effective ligand to form complexes with antifungal activities, followed by carboxymethylated *lentinan* (CLNT) and carboxymethylated *Momordica charantia* polysaccharide (CMCP).

1. Introduction

Rare earths have increasingly been a research focus because of their potential usefulness in the different scientific fields. For example, rare earths are often used as magnetic resonance imaging (MRI) contrast agents (Aime et al., 2006; Picard et al., 2006), as effective catalysts for the hydrolytic cleavage of phosphate ester bonds (Franklin, 2001), and as luminescence probes or active centres for luminescent materials and electroluminescent device (Hemmila & Mukkala, 2001; Pandya, Yu, & Parker, 2006; Regulacio et al., 2008). Rare earth complexes also show peculiar properties such as anticancer and antifungal activities due to their direct cytotoxicity (Fricker, 2006; Sharma, Thripathi, Kanna, & Sharma, 1981). On the other hand, various polysaccharides that are derived from natural sources can stimulate or enhance the host immune systems so that the hosts can have antitumour and antifungi bioactivities. (Chen et al., 2012; Leung, Liu, Koon, & Fung. 2006; Ni et al., 2010; Sun et al., 2013; Zhang, Cui, Cheung, & Wang, 2007).

The rare earth complexes with polysaccharides are expected to combine both the functions of direct cytotoxic activities of rare earths and host immune enhancements of polysaccharides. They may be potentially developed to be a new kind of fungicide with less toxicity and damage to environment and human wellbeing. However, it is challenging to directly form rare earth complexes with polysaccharides, which had been confirmed by our test experiments that precipitates will be certainly produced when even a small amount of rare earth ions ($RECl_3$) are directly mixed with polysaccharide solution. This is because rare earth ions in aqueous solution can form aquated complexes, and the polysaccharides to be added are difficult to compete against the water molecules present in large quantities in aqueous media (Singh, Sharma, & Singh, 2010). Furthermore, displacements of coordinated water molecules by polysaccharides which are a kind of macromolecules through O atoms in —OH groups are nearly impossible. Thus, only strong chelating ligands could form water soluble rare earth complexes with sufficient stability. To our knowledge, carboxyl is an effective group to chelate rare earths and carboxymethylation is a helpful approach used to add carboxyl groups into polysaccharides. Moreover, carboxymethylation can enhance the water solubility of polysaccharides, and it can also change the chain conformation to improve their biological activities (Wang, Zhang, Yu, & Cheung, 2009; Xu et al., 2009). Hence, it is expected that water-soluble rare earth complexes with polysaccharides can be synthesized by chelating coordination of carboxyl groups added into the chains of polysaccharides using carboxymethylation.

In the present study, the natural polysaccharides were extracted from medicinal fungi of *Ganoderma applanatum* fermentation broth, *Lentinus edodes* fruit bodies and plant fruit of *Momordica*

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charantia, known as *Ganoderma applanatum* polysaccharide (GAP), lentinan (LNT) and *Momordica charantia* polysaccharide (MCP), respectively. The three different polysaccharides were carboxymethylated using chloroacetic acid under strong base condition, that is, CGAP, CLNP and CMCP. La, Eu and Yb were selected to represent light, middle and heavy rare earths to individually form complexes with such carboxymethylated polysaccharides. Finally, the antifungal activities of these complexes against five kinds of plant pathogenic fungi were investigated.

2. Experimental

2.1. Materials

The hydrated rare earth (III) chlorides were prepared by dissolving RE_2O_3 (RE: La, Eu and Yb) in concentrated HCl. All chemicals used were of analytical grade.

Valsa mali Miyabe et Yamada (*V. mali*), *Fusarium oxysporum f. sp. vasinfectum* (*F. oxysporum*), *Gaeumannomyces graminis* (*G. graminis*), *Colletotrichum gloeosporioides* (*C. gloeosporioides*) *and Alternaria brassicae* (*A. brassicae*) were used as the test fungal strains.

2.1.1. Isolation of Ganoderma applanatum polysaccharide (GAP)

The strain of *Ganoderma lucidum* was obtained from Shandong Provincial Key Laboratory of Applied Mycology, China. The cultivation was carried out in a 50 L fermentor (DS-Y-50L, Senda Co., Ltd, China) with 10% (v/v) liquid spawn for 6 days at 25 °C. After the removal of mycelia, the supernatant was concentrated in a rotary evaporator and mixed with ethanol (supernatant/ethanol, 1:4, v/v) for 24 h at 4 °C to get complete precipitation (Zhang, Liu, Park, Xia, & Kim, 2012). The precipitates were recovered by centrifugation at 4000 r/min for 20 min and then dialyzed against distilled water for 3 days and freeze dried.

2.1.2. Isolation of Lentinan (LNT) and Momordica charantia polysaccharide (MCP)

Based on the prior method (Kim, Park, Nam, Lee, & Lee, 2003), LNP and MCP were extracted from *Lentinus edodes* and *Momordica charantia* that were purchased from a local market. The homogenized fruit bodies (10 g) of *Lentinus edodes* and *Momordica charantia* were individually suspended in distilled water (200 mL) under agitation at 90 °C for 1 h and then filtered. After the filtrates were concentrated in a rotary evaporator, they were mixed with four volumes of ethanol for 24 h at 4 °C, and then followed by centrifugation at 4000 r/min for 20 min. The precipitates were then dialyzed against distilled water for 3 days and freeze dried. The final products were LNT and MCP.

2.1.3. Preparation of carboxymethylated polysaccharides

Built on the previous method (Bao, Duan, Fang, & Fang, 2001), the polysaccharides (GAP, LNP and MCP, 1.0 g) were individually mixed with 2-propanol (30 mL) and stirred for 6 h at the room temperature. A total of 40.0 mL 60% (m/V) NaOH was added slowly and vigorously stirred for 90 min. Then, chloroacetic acid (60%, 60 mL) was dropwise added into the mixture within 30 min, which was stirred at 65 °C for another 4 h. After the resulting product was cooled and neutralized with HCl, it was dialyzed against distilled water for 4 days and freeze dried. Accordingly, the carboxymethylated polysaccharides were produced as CGAP, CLNP and CMCP, respectively.

2.1.4. Syntheses of rare earth complexes with carboxymethylated polysaccharides

A total of 1.0 g carboxymethylated polysaccharide was dissolved in 80.0 mL distilled water. The rare earth chlorides ($LaCl_3$, $EuCl_3$ and $YbCl_3$, 0.02 mol/mL) were added dropwise with vigorous agitation individually until few precipitates appeared in the solutions. During the whole process, the solution pH was maintained at 7.0. After an additional 24 h, the resulting solutions were concentrated in a rotary evaporator and freeze dried. Thus, three kinds of rare earth element complexes with three carboxymethylated polysaccharides were obtained as RE–CGAP, RE–CLNP and RE–CMCP (RE: La, Eu and Yb).

2.2. Structural characterization

Fourier transform infrared (FT-IR) spectra were recorded in the range of 4000–500 cm^{-1} using a Thermo Electron FT-IR spectrometer (IR200) at the room temperature. Samples in solid state were ground in the form of KBr discs at a rate of 1:20. The ^{13}C NMR spectra were recorded using a Bruker DSX-300 spectrometer at 30 °C with the freeze-dried samples dissolved in 99.96% D_2O in a 5-mm tube.

2.3. Assays for antifungal activities of the rare earth complexes

Growth rate method was used to perform assays for antifungal activities. Mycelial discs (6 mm in diameter) of the test plant pathogenic fungi were placed in the centre of sterile petri dishes (60 mm × 15 mm) which contained 10 mL potato dextrose agar (PDA) and the rare earth complexes with carboxymethylated polysaccharides at five different concentrations. Distilled water was used as the control contained in PDA. The plates were incubated at 27 ± 2 °C till the mycelia colonies of the control group reached the edge of the plates, and then mycelia growth with PDA containing rare earth–polysaccharide complexes were determined by measuring the colony diameter using decussating method. Each treatment was replicated three times. Radial growth inhibition percentage (%) = $(A_0 - A)/A_0 \times 100\%$ was calculated to assess antifungal activities of the rare earth complexes with carboxymethylated polysaccharides, where A_0 was the mycelia growth diameter of the control group and A was the mycelia growth diameter of rare earth complex treated group.

Toxicity regression equations of logarithm of complexes concentration—growth inhibition percentage were calculated in Excel, through which the half maximal effective concentration (EC_{50}), as a key index for inhibitory activity, was also obtained.

3. Results and discussion

3.1. Structural characterization

Fig. 1 presents the IR spectra of GAP, CGAP and Yb–CGAP. Other polysaccharides and complexes showed similar IR data, so they are not exhibited here. The spectrum of GAP demonstrated a typical signal of a polysaccharide in the range from 4000 cm^{-1} to 500 cm^{-1}. The predominant band centred at 3467 cm^{-1} was attributed to the stretching vibration of OH groups. The peak at 2936 cm^{-1} was assigned to the stretching vibration of the CH_2 groups while the symmetrical deformation vibration of the CH_2 groups was observed at the absorption of 1447 cm^{-1}. There was a vibration band corresponding to the associated water in the region of 1654 cm^{-1}. The peaks at 1159 cm^{-1} and 1030 cm^{-1} belonged to asymmetrical and symmetrical stretching vibrations of C—O—C, respectively. After carboxymethylation, two new intensive absorption bands corresponding to the asymmetrical and symmetrical stretching vibrations of COO^- group were observed at 1623 cm^{-1} and 1333 cm^{-1}, which provided a strong evidence of carboxymethylation of the polysaccharide.

On coordination, sodium carboxylates of carboxymethylated polysaccharide were converted into rare earths coordination

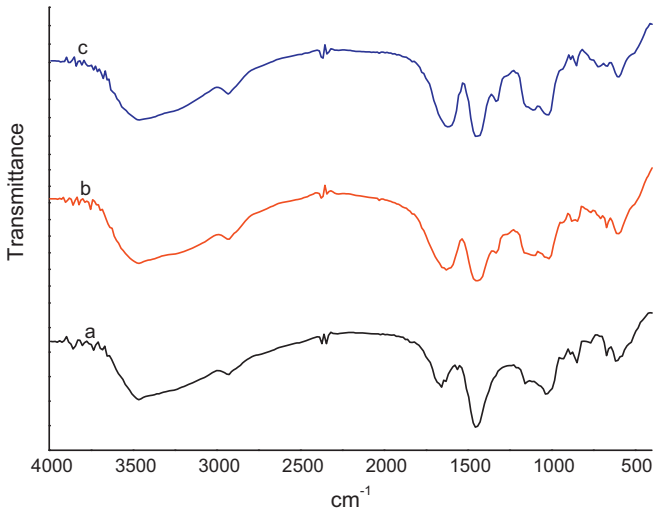

Fig. 1. IR spectra of (a) GLP, (b) CGAP and (c) Yb–CGAP.

complexes, and the asymmetrical and symmetrical stretching vibration were shifted to 1607 cm^{-1} and 1325 cm^{-1} (Fig. 1). The observed difference $\Delta\nu(COO^-)$ between the positions of the $\nu_{as}(COO^-)$ and $\nu_s(COO^-)$ in rare earth complex was 382 cm^{-1} which was similar to that in sodium compound, indicating that carboxylates appeared to be chelating groups.

The NMR spectra of GAP, CGAP and Yb–CGAP provided more detailed structure information, as shown in Fig. 2. Compared to the native polysaccharide GAP, a new resonance peak appeared at 176.90 ppm in the NMR spectrum of the carboxymethylated polysaccharide, which was assigned to the carboxylate group and also presented a further evidence for the carboxymethyl substitution. In the rare earth complex, the resonance signal of carboxylate group at 176.90 ppm nearly disappeared, suggesting that the rare earth element was coordinated by carboxymethylated polysaccharide through carboxylate groups.

3.2. Antifungal effects of the rare earth complexes with carboxymethylated polysaccharides against plant pathogenic fungi

The inhibition effects of the rare earth complexes with carboxymethylated polysaccharides were evaluated against five kinds of plant pathogenic fungi (i.e. *V. mali, F. oxysporum, G. graminis, C. gloeosporioides* and *A. brassicae*) using growth rate method. These rare earth complexes exhibited various antifungal activities towards the tested fungi depending on rare earth elements, polysaccharide types and fungal species.

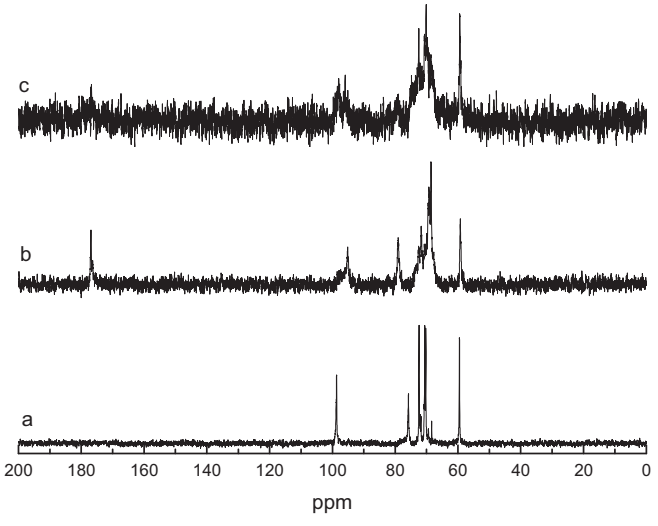

Fig. 2. ^{13}C NMR spectra of (a) GAP, (b) CGAP and (c) Yb–CGAP.

As shown in Fig. 3, the La–CGAP complex had a significant dose-dependent antifungal effect against *V. mali, F. oxysporum, G. graminis* and *C. gloeosporioides*. The radial growth inhibition percentages of La–CGAP against these tested plant pathogenic fungi ranged from 40.16 to 56.06% at the maximum concentration of 6.25 mg/mL. This complex clearly exhibited a linear relationship between logarithm concentration and radial growth inhibition (Table 1). Among the five tested plant pathogenic fungi, the La–CGAP complex revealed the strongest antifungal effect against *C. gloeosporioides* with EC_{50} of 4.10 mg/mL. However, the La–CGAP complex exhibited the weakest inhibition towards *A. brassicae* with dose independence. Its radial growth inhibition percentage was low and remained stable when the complex concentration increased.

Eu was selected to represent the middle rare earth elements to form complex with carboxymethylated polysaccharides in the present study. Its antifungal activity was also investigated against the plant pathogenic fungi using growth rate method. In general, the Eu–CGAP complex exhibited less radial growth inhibition against these fungi than the La–CGAP complex, as demonstrated in Fig. 3. Among the five tested fungi, the Eu–CGAP complex showed the highest antifungal effect against *G. graminis*, but with a large EC_{50} of 9.53 mg/mL.

Obviously, the heavy rare earth complex, Yb–CGAP, revealed promising antifungal effects against all these five tested plant pathogenic fungi with dose dependence. High inhibition percentages and low EC_{50} values of Yb–CGAP were observed, as presented in Fig. 3 and Table 1. For instance, at the concentration of 6.5 mg/mL, the radial growth inhibition percentages of this complex were significantly high, ranging from 78.7 to 90.40%. The most effective antifungal activity of Yb–CGAP was observed towards *G. graminis* with a considerably low EC_{50} value of 1.06 mg/mL. As La–CGAP and Eu–CGAP, Yb–CGAP also showed the weakest antifungal effect towards *A. brassicae* but with a low EC_{50} value of 4.04 mg/mL, which was much less than that of La–CGAP and Eu–CGAP against any fungi tested. Thus, it suggested that Yb–CGAP was much more effective than La–CGAP and Eu–CGAP against all the tested plant pathogenic fungi (Fig. 3 and Table 1).

In brief, Yb–CGAP, as a heavy rare earth contained complex, exhibited the highest inhibition activity. The inhibition effect of the light rare earth contained complex (La–CGAP) was higher than that of the middle rare earth contained complex (Eu–CGAP). It can be concluded that metal elements played a key role in cytotoxicity to the tested fungi. More specifically, the La–CGAP showed the highest inhibition effect towards *C. gloeosporioides* while Eu–CGLP and Yb–CGAP revealed the highest inhibition effect both towards *G. graminis*. All the rare earth complexes with CGAP exerted the lowest inhibition against *A. brassicae*. Hence, the rare earth complexes with CGAP exhibited selective inhibition depending on fungal species.

In order to assess the effect of ligand type on antifungal activity, another polysaccharide extracted from *Lentinus edodes* which is one of medicinal fungi was employed to form rare earth complexes after carboxymethylation (RE–CLNT). The antifungal activities of RE–CLNT were assessed using the same plant pathogenic fungi, as mentioned above (Fig. 4 and Table 2). The inhibition effects of RE–CLNT against the tested fungi were found to be significantly different from that of RE–CGAP.

The inhibition percentages of La–CLNT against all the tested fungi were less than 40% at the maximum concentration of 6.92 mg/mL, which were much lower than that of La–CGAP (Fig. 4). All the EC_{50} values of La–CLNT were found to be higher than 10 mg/mL (Table 2). As a result, the La–CLNT complex exhibited

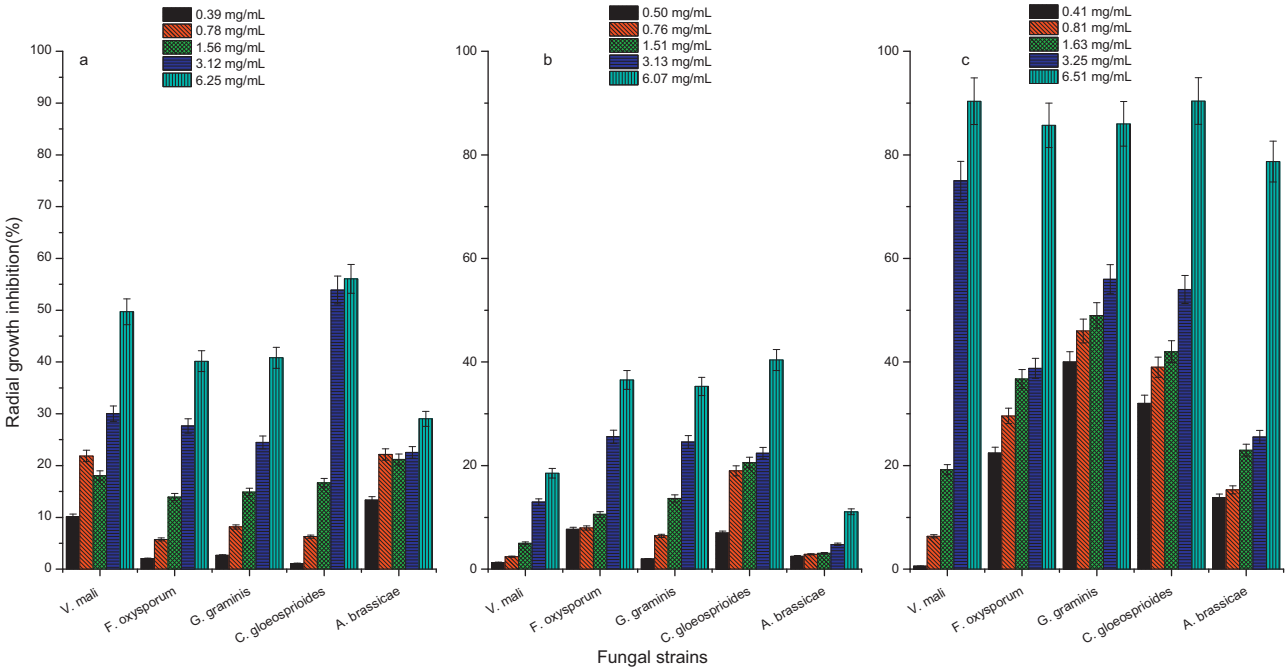

Fig. 3. Antifungal effect of RE–CGAP ((a) La–CGAP, (b) Eu–CGAP, (c) Yb–CGAP) on plant pathogenic fungi.

Table 1
Statistical results of RE–CGAP antifungal activities against plant pathogenic fungi.

Coordination compounds	Fungus species	Virulence equation	Correlation coefficient (r)	EC_{50} (mg/mL)	95% Confidence limit (mg/mL)	
					Lower	Upper
La–CGLP	*V. mali*	Y = 4.1218 + 0.9252X	0.9334	8.80	0.30	266.86
	F. oxysporum	Y = 3.5960 + 1.5193X	0.9241	8.40	0.84	84.09
	G. graminis	Y = 3.6768 + 1.3646X	0.9968	9.32	0.68	128.10
	C. gloeosprioides	Y = 3.6702 + 2.1685X	0.9778	4.10	1.12	14.99
	A. brassicae	Y = 4.1303 + 0.3751X	0.8902	208.31	0	–
Eu–CGLP	*V. mali*	Y = 3.1640 + 1.2623X	0.9958	28.48	0.29	2826.20
	F. oxysporum	Y = 3.7563 + 1.0773X	0.9649	14.27	0.35	575.18
	G. graminis	Y = 3.5423 + 1.4886X	0.9836	9.53	0.81	111.67
	C. gloeosprioides	Y = 3.9785 + 0.8992X	0.9053	13.68	0.17	1096.25
	A. brassicae	Y = 3.1453 + 0.6272X	0.9158	906.20	0	–
Yb–CGLP	*V. mali*	Y = 3.7184 + 3.2763X	0.9916	2.46	1.12	5.41
	F. oxysporum	Y = 4.5559 + 1.2961X	0.8611	2.20	0.60	8.11
	G. graminis	Y = 4.9754 + 0.9699X	0.8794	1.06	0.15	7.61
	C. gloeosprioides	Y = 4.8154 + 1.3041X	0.8758	1.39	0.38	5.08
	A. brassicae	Y = 4.1671 + 1.3739X	0.8486	4.04	0.73	22.32

Table 2
Statistical results of RE–CLNP antifungal activities against plant pathogenic fungi.

Coordination compounds	Fungus species	Virulence equation	Correlation coefficient (r)	EC_{50} (mg/mL)	95% Confidence limit (mg/mL)	
					Lower	Upper
La–CLNT	*V. mali*	Y = 4.2061 + 0.4599X	0.9427	53.22	0.001	–
	F. oxysporum	Y = 3.6599 + 1.2553X	0.9942	11.68	0.55	249.41
	G. graminis	Y = 3.6061 + 1.3157X	0.9858	11.46	0.62	212.58
	C. gloeosprioides	Y = 3.6510 + 0.9064X	0.9646	30.78	0.10	9072.94
	A. brassicae	Y = 3.5453 + 0.3199X	0.9409	–	0	–
Eu–CLNT	*V. mali*	Y = 3.4643 + 0.8757X	0.9479	56.71	0.06	–
	F. oxysporum	Y = 3.4063 + 1.6417X	0.9456	9.35	1.04	84.13
	G. graminis	Y = 3.5296 + 1.2827X	0.9856	14.01	0.62	314.24
	C. gloeosprioides	Y = 3.4834 + 0.8688X	0.8785	55.67	0.061	–
	A. brassicae	Y = 3.3515 + 0.5985X	0.9917	568.30	0	–
Yb–CLNT	*V. mali*	Y = 4.1687 + 3.1245X	0.9890	1.85	0.99	3.43
	F. oxysporum	Y = 3.5865 + 1.3699X	0.8746	10.76	0.65	177.88
	G. graminis	Y = 3.5220 + 1.5034X	0.8530	9.62	0.70	132.70
	C. gloeosprioides	Y = 3.6248 + 2.0503X	0.9804	4.69	1.14	19.34
	A. brassicae	Y = 3.0502 + 0.4536X	0.8785	–	0	–

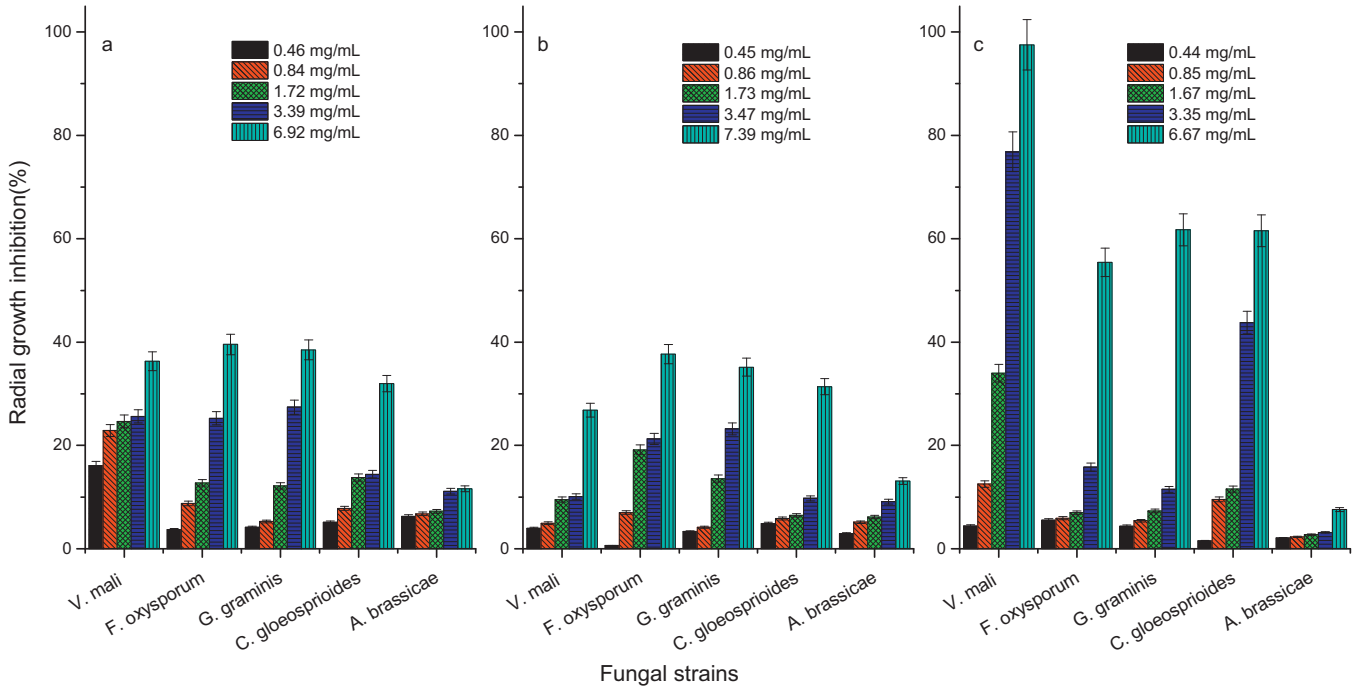

Fig. 4. Antifungal effect of RE–CLNT ((a) La–CGAP, (b) Eu–CGAP, (c) Yb–CGAP) on plant pathogenic fungi.

Table 3
Statistical results of Yb–CMCP antifungal activities against plant pathogenic fungi.

Coordination compounds	Fungus species	Virulence equation	Correlation coefficient (r)	EC_{50} (mg/mL)	95% Confidence limit (mg/mL)	
					Lower	Upper
Yb–CMCP	*V. mali*	Y = 3.3997 + 2.1971X	0.9496	5.3505	1.247	22.963
	F. oxysporum	Y = 3.7387 + 0.3314X	0.9792	–	0	–
	G. graminis	Y = 3.5761 + 0.9611X	0.8993	30.3072	0.136	6741.847
	C. gloeosprioides	Y = 3.8003 + 1.3516X	0.9485	7.7199	0.805	74.003
	A. brassicae	Y = 3.2919 + 0.2085X	0.9549	–	0	–

lower antifungal effects against the tested fungi than La–CGAP. Furthermore, the inhibition activity of Eu–CLNT against the tested fungi with higher EC_{50} values and lower inhibition percentages was found to be less effective than La–CLNT and Eu–CGAP.

Similar to CGAP, the complex containing Yb showed the most effective inhibition against *V. mali, F. oxysporum, G. graminis* and *C. gloeosporioides* among all the rare earth complexes with CLNT; Yb-CLNT also exhibited remarkably high inhibition percentages against *V. mali* (97.49%), *F. oxysporum* (55.44%), *G. graminis* (61.72%) and *C. gloeosporioides* (61.56%) at the concentration of 6.67 mg/mL. But its EC_{50} values varied, ranging from 1.85 mg/mL to 10.77 mg/mL. Apparently, the lowest antifungal effects of all the three rare earth complexes with CLNT were also observed towards *A. brassicae*. In general, Yb–CLNT was found to be less effective against the tested fungi than Yb–CGAP. Thus, ligand type had a significant impact on antifungal activities of rare earth–carboxymethylated polysaccharide complexes.

In order to further investigate the effect of ligand on antifungal activity, CMCP was used as another type of ligand to form rare earth complex. Here, MCP was extracted from plant fruit, *Momordica charantia* while the GAP and LNT were from medicinal fungi. Considering that Yb was the best rare earth element to form the effective complexes with carboxymethylated polysaccharides to inhibit the tested fungi, only the antifungal activity of Yb–CMCP was then assessed to further examine the effect of ligand on inhibition effectiveness of rare earth complexes. As shown in Fig. 5 and Table 3, Yb–CMCP revealed potential inhibition against

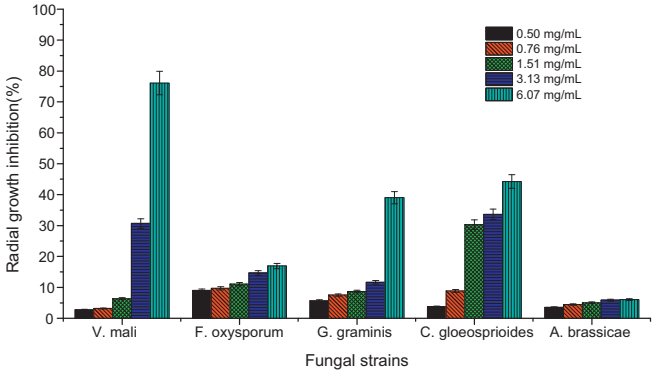

Fig. 5. Antifungal effect of Yb–CMCP on plant pathogenic fungi.

V. mali and *C. gloeosporioides* with EC_{50} values being 5.35 mg/mL and 7.71 mg/mL, respectively, and the inhibition percentages were 76.11% and 44.25% at the maximum concentration of 8.21 mg/mL. Nevertheless, Yb–CMCP exhibited considerably weak antifungal effect against *G. graminis, F. oxysporum* and *A. brassicae*. Generally, the Yb–CMCP complex exhibited lower antifungal effects against the tested fungi than Yb–CGAP and Yb–CLNT, which further indicated that ligands also played a key role in promoting cytotoxicity of rare earth complexes towards tested fungi.

4. Conclusion

The IR and NMR data confirmed that rare earth elements successfully formed complexes with polysaccharides by chelating of carboxyl groups added by means of carboxymethylation. The rare earth complexes showed potential antifungal properties, but the inhibition ability of these complexes depended on metal elements, ligand types and fungi species. The rare earth complexes exerted various and selective inhibitions against the plant pathogenic fungi. Among all the synthesized rare earth complexes, Yb complexes showed the most effective antifungal activities. For ligands, CGAP promoted cytotoxicity of the complexes most effectively. Additionally, GAP can be obtained from fermentation broth in bulk. Therefore, Yb–CGAP can potentially be developed as a kind of promising fungicide.

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