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The complexes of cannabidiol mediated by bridged cyclodextrins dimers with high solubilization, in vitro antioxidant activity and cytotoxicity

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ABSTRACT

The poor water-solubility of Cannabidiol (CBD) seriously hinders its pharmacological activities such as anti-anxiety, anti-epilepsy, anti-oxidation and anti-cancer, etc. In this paper, we successfully designed and synthesized two bridged cyclodextrins (CDs) dimers with different length of bridged chains (succinic acid (SACDD) and 3,3′-thiodipropionic acid (TPACDD)) to encapsulate CBD, a reported DMCD (2,6-Di-O-methyl-β-cyclodextrin) with single cavity was used as a control complex. Their characteristics and inclusion complexation behaviors were investigated via XRD, SEM, NMR and TGA, the obtained data suggests CBD is successfully encapsulated into two cavity of bridged CD dimers with stronger stability constants, compared with DMCD. Water solubility of CBD is significantly promoted by 6.76×10^5 and 4.52×10^5 folds after formation of inclusion complexes, as well as the antioxidant activity in vitro (5.26-fold enhanced). MTT assays shows they remained effective anti-tumor activity, while they barely show cytotoxicity to normal cell. Our work might provide a strategy for development and application of water-solubility CBD.

1. Introduction

It is well known that cannabis is a strictly controlled in the United Nations drug conventions, so its application is seriously restricted. Two main active components of cannabis (Tetrahydrocannabinol (THC) and CBD (Fig. 1)) are isomers, but their effect are quite different [1,2]. THC is considered as drugs due to its hallucinogenic effects and addictive properties, while CBD is not addictive and can inhibit the side effects of THC [3,4]. In recent years, with the wave of legalization of CBD, CBD has become a star molecule for scientists to study. Numerous studies have revealed that CBD shows a variety of pharmacological activities, such as anti-anxiety, anti-epilepsy, anti-oxidation, anti-cancer, etc. [5–7]. The first CBD drug Epidiolex® for the therapy of epilepsy was approved by FDA in June 2018 [8]. Recently, Cannabis Pharmaceuticals in the United States announced that their first patent candidate cannabinoid anticancer drug "RCC-33" shows good efficacy in nude mice with colorectal cancer [9]. However, poor water-solubility and low bioavailability of CBD greatly limit its further development in the application of biomedicine. Therefore, the development of soluble CBD has become an urgent issue to be solved in the cannabis industry.

At present, among the general methods of preparing soluble CBD in the world, the "Trait Distilled™" technology was link a sugar molecule to a hydroxyl group of CBD molecule, which has been identified as a promising development technology of water-soluble CBD [10,11], however, two hydroxyl groups and alkyl chains of CBD's structure are necessary for its biological activity, this method may affect the bioactivity of CBD. Therefore, supramolecular inclusion technology may be an alternative strategy to solve the water-solubility of CBD. It has been reported that Lv et al. used three natural cyclodextrins (CDs: α-CD, β-CD and γ-CD) to improve the water-solubility of CBD by 33493 ~ 84530 folds in 2019 [12]. Lechanteur et al. also used α-CD, β-CD, γ-CD, HP-β-CD, HP-γ-CD and CH_3-β-CD (DMCD, Fig. 1) to improve the water-solubility of CBD, and DMCD could enhanced solubility of CBD by 406699-fold, unfortunately, they did not further study of inclusion behavior of CBD with DMCD [13].

Host-pharmaceutical drug interactions for improving drug solubility and bioavailability have been a hot research topic in recent years [14–17]. CDs are cyclic oligosaccharide compounds, which normally composed of many glucopyranoside monomers linked by α-1,4 glycoside bonds. Because of its excellent water-solubility and safety, it is widely used in the fields of food and drug [18,19]. CDs have a hydrophobic interior cavity and a hydrophilic

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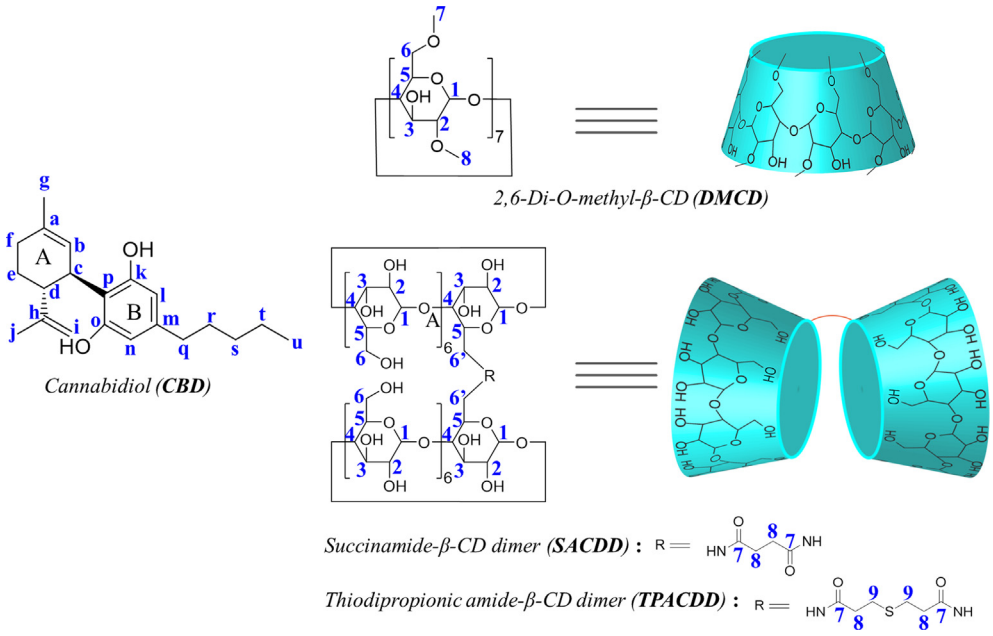

Fig. 1. Structures of CBD, DMCD, SACDD and TPACDD.

exterior surface. The hydrophobic cavity of CDs is able to recognize many guest molecules (organic molecules, inorganic molecules, peptides and proteins, etc.) by non-covalent interactions (hydrophobic interactions, hydrogen bonds and van der Waals forces) to form stable inclusion complexes. However, the binding ability of natural cyclodextrin is relatively weak with guest molecules (Ks < 10^5 M^{-1}), even if with simple structural modification, it's hard to improve the binding ability effectively [20–22]. Therefore, two CDs are linked by bridging chain groups to form bridged CD dimer, the two cavities of bridged CD dimer both participate in the inclusion and coordination of guest molecule to improve their binding ability (Ks > 10^{11} M^{-1}) [23]. Bridged CD was synthesized in early 1970s, and it has been studied and developed by researchers [20,24,25]. Recently, Chen et al. designed and synthesized a pair of β-CDs covalently linked by acid-sensitive arylhydrazone and GSH-sensitive disulfide bonds to achieve a precise drug release pattern [26].

Recently, our group used bridged CD to encapsulate Podophyllotoxin, and the Ks value is 6415 M^{-1}, the water-solubility of Podophyllotoxin was increased by 424-fold [27]. Herein, we designed and synthesized two bridged CD dimer with succinic acid (SACDD, Fig. 1) and 3,3′-thiodipropionic acid (TPACDD, Fig. 1) as bridging chain groups (their chain lengths differ by a factor of two) to encapsulate CBD, and compared with of DMCD-CBD inclusion complex. We predicted that the binding ability of bridged CD to CBD was stronger than that of DMCD, and the water-solubility of CBD could be significantly improved after the inclusion complexes was formed. And we also expect the inclusion complexes has good antioxidant activity and anticancer activity.

2. Experimental

2.1. Materials

All the reagents were purchased from commercial sources and used without further purification. CBD used in this work was from Yunnan Perrrin Technology Co. Ltd. (Kunming, China). 2,6-Di-O-methyl-β-CD (DMCD) used in this work was purchased from Aladdin (Shanghai, China). All other chemicals and solvents were of analytical grade.

2.2. Methods

2.2.1. Synthesis of Mono-(6-deoxy-6-amino)-β-CD (ACD)

Mono-(6-O-tosyl)-β-CD (TsCD) and Mono-(6-deoxy-6-azide)-β-CD (NCD) were synthesized and purified according to previously reported procedures [28]. ACD was synthesized via procedures similar to that in our previous work. Specifically, triphenylphosphine (6.76 g, 25.80 mmol) was added to a stirred solution of NCD (10.00 g, 8.60 mmol) in DMF (150 mL). The resulting solution was stirred at room temperature for 12 h. Then, $NH_3{\cdot}H_2O$ (80 mL) was added to the resulting solution and continue to reacted at room temperature for 18 h. After filtered, the filtrate was precipitated in acetone (2.0 L). White precipitates were collected and washed three times with acetone (800 mL × 3) and dried under vacuum to yield product as white solid (7.32 g, 6.46 mmol, 75%). ^{1}H NMR (600 MHz, D_2O, ppm): δ 4.93 (h, J = 3.4 Hz, 7H, 1-H of β-CD), 3.85–3.66 (m, 26H, 3, 5, 6-H of β-CD), 3.53–3.29 (m, 14H, 2, 4-H of β-CD), 2.99–2.63 (m, 2H).

2.2.2. Synthesis of Succinimide-β-CD dimer (SACDD)

Succinic acid (0.08 g, 0.66 mmol), N, N-Diisopropylethylamine (DIPEA) (0.17 g, 1.32 mmol), O-(7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) (0.50 g, 1.32 mmol) were dissolved in dry DMF (15 mL). The resulting solution was stirred at room temperature for 4 h. Then, ACD (1.00 g, 0.88 mmol) was dissolved in dry DMF (15 mL) and dripped into the above solution. The mixture was stirred roughly at room temperature for 48 h. The mixture was dropped into acetone (200 mL) to precipitate. The precipitate was collected by centrifugation and washed by acetone (100 mL × 3). The resultant was dialyzed (MWCO, 2000) against distilled water for 48 h and then freeze dried for 24 h. The final product was SACDD (0.15 g, 0.064 mmol, 15%). ^{1}H NMR (600 MHz, D_2O, ppm): δ 4.99 (dt, J = 10.9, 5.4 Hz, 14H, 1-H of β-CD), 3.93–3.73 (m, 52H, 3, 5, 6-H of β-CD), 3.70–3.45 (m, 28H, 2, 4-H of β-CD), 3.32 (dt, J = 38.6, 11.8 Hz, 4H, 6′-H of β-CD), 2.61–2.35 (m, 4H, 8-H of β-CD). ESI-MS: m/z calcd. for $[M + H]^+$: 2350.7803, found: 2350.7825.

2.2.3. Synthesis of thiodipropionic amide-β-CD dimer (TPACDD)

3,3′-Thiodipropionic acid (0.12 g, 0.66 mmol), N, N-Diisopropylethylamine (DIPEA) (0.17 g, 1.32 mmol), O-(7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) (0.50 g, 1.32 mmol) were dissolved in dry DMF (15 mL). The resulting solution was stirred at room temperature for 4 h. Then, ACD (1.00 g, 0.88 mmol) was dissolved in dry DMF (15 mL) and dripped into the above solution. The mixture was stirred roughly at room temperature for 48 h. The mixture was dropped into acetone (200 mL) to precipitate. The precipitate was collected by centrifugation and washed by acetone (100 mL × 3). The resultant was dialyzed (MWCO, 2000) against distilled water for 48 h and then freeze dried for 24 h. The final product was TPACDD (0.19 g, 0.079 mmol, 18%). ^{1}H NMR (600 MHz, D_2O, ppm): δ 5.00 (dt, J = 8.9, 5.4 Hz, 14H, 1-H of β-CD), 3.95–3.71 (m, 52H, 3, 5, 6-H of β-CD), 3.60–3.27 (m, 28H, 2, 4-H of β-CD), 2.93–2.18 (m, 14H, 6′, 9, 8-H). ESI-MS: m/z calcd. for $[M + H]^+$: 2409.7803, found: 2409.7884.

2.2.4. Preparation of CDs-CBD inclusion complexes

Solid inclusion complexes were prepared by saturated aqueous solution method. Accurately weighed amounts of DMCD, SACDD and TPACDD (0.1 mmol) were dissolved in ultrapure water (20 mL), respectively. Subsequently, CBD (0.2 mmol) was added to the above solution, respectively. The resulting suspension was stirred in the dark at room temperature for 72 h. It was then filtered through a 0.45-μm membrane filter and the filtrate was dried in vacuum freeze dryer to yield CDs-CBD solid inclusion complexes.

2.2.5. Preparation of CDs-CBD physical mixtures

The physical mixtures were prepared by mixing the powders in a 1:1 M ratio of CBD and CDs (DMCD, SACDD and TPACDD) in an agate mortar for 5 min, respectively.

2.2.6. Phase-solubility diagram

The phase-solubility diagram was performed according to the studies of Higuchi and Connors [29]. Excess amounts of CBD were added in solutions with increasing concentration of CDs (DMCD, SACDD and TPACDD), range in 1.0 to 7.2 mM, respectively. The samples were sealed and were shaken for 72 h at room temperature in the dark. Then, they were filtered on 0.45 μm membranes. The filtered samples were suitable diluted and analyzed in a Shimadzu® UV–Vis spectrophotometer (UV-2250) for dissolved concentration of CBD at 274 nm. All experiments were performed in triplicates and the phase-solubility diagram was drawn by plotting the molar concentration of CBD against the molar concentration of CDs (DMCD, SACDD and TPACDD) according to the calibration curve.

2.2.7. Powder X-ray diffractometry (XRD)

The X-ray powder diffraction patterns were obtained with an XRD-6000 X-ray Diffractometer (Shimadzu, Japan) using a Ni-filtered, Cu Kα radiation, a voltage of 40 kV and a 30 mA current. the physical mixture of CBD and CDs (DMCD, SACDD and TPACDD) and their inclusion complex were previously dried for 24 h at 110 °C. Each dried powder was measured in the 2θ angle range between 5° and 60° with a scan rate of 5° min^{-1} and a step size of 0.02°.

2.2.8. Scanning electron microscope (SEM) analysis

SEM analysis was carried out with a Jeol JSM-840 scanning electron microscope (Japan). The samples were mounted on metal stubs with double-sided adhesive tapes. To avoid the issue of electrically charging from the insulating samples, a thin layer of gold was sputtered on top of the samples prior to the SEM scan. The micrographs were then obtained with an accelerating potential of 15 kV under reduced pressure.

2.2.9. Nuclear magnetic resonance (NMR) spectroscopy

All NMR analyses including ^{1}H and 2D ROESY NMR experiments were conducted on a Bruker Avance III HD spectrometer (600 MHz, Bruker BioSpin, Switzerland) at 25 °C. The samples were dissolved in 99.98% D_2O or 99.98% $CDCl_3$ and were filtered before use.

2.2.10. Water solubility test

The water solubility of CDs-CBD inclusion complexes was assessed by preparation of their saturated solution. Briefly, excess amounts of CDs-CBD inclusion complexes were placed in buffer solution (KH_2PO_4/K_2HPO_4, pH 7.0, 2 mL) and shaken at 25 °C for 2 h, respectively. The mixture was then filtered through 0.45-μm membrane to obtain a clear solution. The obtained solution was suitable diluted and measured using Shimadzu® UV–Vis spectrophotometer (UV-2250) under 274 nm. The water solubility of CDs-CBD inclusion complexes could be deduced from the standard curve of CBD.

2.2.11. Thermal gravimetric analysis (TGA)

The TGA curves of CDs, CBD, CDs-CBD physical mixtures and inclusion complexes were performed on a simultaneous thermal analyzer (NETZSCH STA449F3 Germany). About 2 mg of the sample was placed in an aluminum crucible and subjected to a temperature range of 40 ~ 400 °C, under dynamic nitrogen atmosphere (50 mL·min^{-1}) and heating rate of 10 °C·min^{-1}.

2.2.12. In vitro antioxidant assays

The DPPH (2, 2-diphenyl-1-picrylhydeazyl) scavenging assay was used to evaluate the in vitro antioxidant activity of CDs-CBD inclusion complexes [30,31]. Firstly, the stock solutions of CBD, CDs and three inclusion complexes (0.5 mM, 1.0 mM, 1.5 mM, 3.0 mM and 5.0 mM, 10.0 mL) were prepared by ethanol and water respectively. Then a volume of 50 μL of stock solutions was mixed with 150 μL of DPPH in ethanol (0.2 mM) in a 96-well plate. After being cultivated at room temperature for 30 min in the dark, they were subjected to the absorbance determination on Enzyme-labeled instrument at 517 nm. The free radical scavenging activity of the sample could be expressed in the percentage of remaining DPPH (% $DPPH_{rem}$) which could be calculated from Eq. (1).

$$\%DPPH_{rem} = \frac{A_{A(t)}}{A_{C(0)}} \times 100\% \quad (1)$$

where $A_{C(0)}$ and $A_{A(t)}$ are the initial and the final absorbance of DPPH, respectively. All tests were run in triplicate.

EC_{50} represents the concentration of the inhibitory effect of the antioxidant when the clearance rate is 50%, which is used to evaluate the antioxidant capacity of the antioxidant. Under the same conditions, the lower the EC_{50} value, the stronger the antioxidant capacity. All experiments were carried out in triplicate, and the average value was calculated.

2.2.13. In vitro cytotoxicity assays

The in vitro cytotoxicity of solid inclusion complexes was evaluated towards two human cancer cell lines, MCF-7, HT29 and normal cell line HLF, using cisplatin as a positive reference by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [32,33]. Cells were suspended in RPMI 1640 (Hyclone Corp. Utah, USA) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Afterward, cells were seeded into 96-well microculture plates. Culture for 24 h, CBD, DMCD, SACDD, TPACDD and their inclusion complex were added, respectively. After 48 h exposure to the compounds,

cells viability was determined by the MTT cytotoxicity assay, recording the absorbance at 490 nm by a microplate spectrophotometer. All experiments were conducted triplicate.

3. Result and discussion

3.1. Phase-solubility diagram analysis

Phase-solubility diagram of SACDD-CBD, TPACDD-CBD and DMCD-CBD inclusion complexes are shown in Fig. 2. the solubility of CBD was enhanced with increasing CDs (SACDD, TPACDD and DMCD) concentration, it shows the phase-solubility curves of three inclusion complexes are A-type diagrams. Besides, SACDD-CBD, TPACDD-CBD and DMCD-CBD inclusion complexes showed linear increase in phase-solubility curves. According to Higuchi and Connors's theory, the phase-solubility curves of SACDD-CBD and TPACDD-CBD are classified as a typical A_L-type, they have formed 1:1 inclusion complex [34]. For DMCD-CBD, according to the previous studies by Lechanteur, DMCD-CBD also can form 1:2 inclusion complex, when DMCD concentration was increased to 150 mM, the phase-solubility curve is classified as a typical A_P-type [35–38].

In order to further compare the binding ability of three CDs (SACDD, TPACDD and DMCD) with CBD, we studied the case of their 1:1 inclusion complex. Besides, we also compared with the natural CDs (α-CD, β-CD and γ-CD) previously studied by Lv et al [12]. The binding constants (*Ks*) of SACDD-CBD, TPACDD-CBD and DMCD-CBD inclusion complexes were calculated by the phase-solubility diagram according to the equation (2).

$$Ks = \frac{Slope}{S_0(1 - Slope)} \quad (2)$$

Where slope is the value obtained in the linear regression and S_0 is the aqueous solubility of the CBD at pH 7.0 ($S_0 = 1.994 \times 10^{-7}$ mol·L^{-1}) in the absence of CDs [12].

Table 1 presents the stability constant (*Ks* and log *Ks*) and Gibbs free energy change ($-\Delta G^0$) of different CDs and CBD calculated by phase-solubility diagram. For several common CDs (α-CD, β-CD, γ-CD and DMCD) with a single cavity, the binding constant of DMCD and CBD is maximum ($Ks = 3.279 \times 10^5$ M^{-1}), and the binding constants of α-, β- and γ-CD are only between 300 ~ 1500 M^{-1}. As the cavity size is the same for DMCD and β-CD, the higher binding constants may reflect that the methyl group (CH_3) of DMCD (relative to β-CD) may provide a stronger hydrophobic driving force for complexation. Bis (β- CD) both has higher binding ability toward CBD than DMCD, their binding constants of two bis (β-CD) (SACDD and TPACDD) with CBD are 3.353×10^6 and 7.540×10^5 M^{-1}, respectively. So, we may predict that the bis (β-CD) with shorter bridged chain has a higher binding ability toward CBD.

3.2. Powder XRD analysis

The powder XRD patterns of CDs (SACDD, TPACDD and DMCD), CBD, CDs-CBD physical mixture (1:1) and CDs-CBD inclusion complexes are shown in Fig. 3, SACDD (Fig. 3a) shows amorphous pattern with two broad peaks at 12.2° and 18.7°, while CBD (Fig. 3b) displayed a series of sharp diffraction peaks at 2θ = 9.7°, 10.2°, 13.2°, 14.9°, 17.3°, 18.8° and 22.1°. The powder XRD pattern of physical mixture of SACDD and CBD (Fig. 3c) displayed a simple superposition of SACDD and CBD, and retained some characteristic crystal peaks of CBD. The characteristic crystal peaks of CBD disappear completely in the powder XRD pattern SACDD-CBD inclusion complex (Fig. 3d), and it is similar to the powder XRD pattern SACDD (Fig. 3a). These results indicates that SACDD and CBD formed inclusion complex. Besides, similar results are obtained for powder XRD patterns of TPACDD-CBD (Fig. S8) and DMCD-CBD (Fig. S9), which also suggests that they formed inclusion complexes.

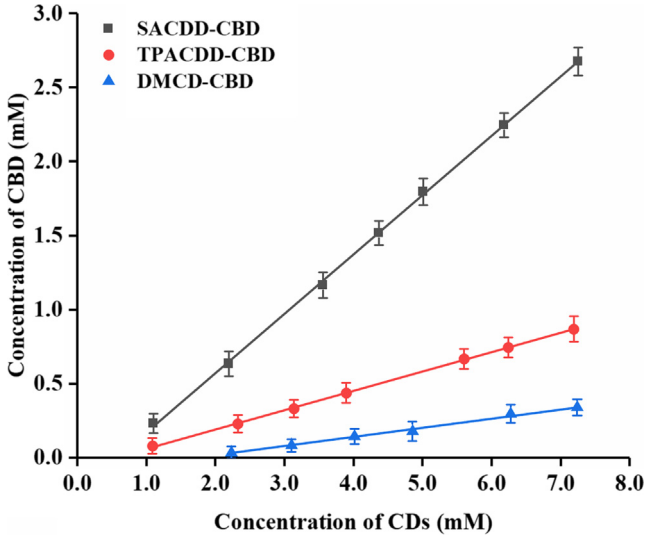

Fig. 2. Phase-solubility diagram of CBD in KH_2PO_4/K_2HPO_4 buffer solution (pH 7.0, 25 °C) depending on the CDs concentration (SACDD, TPACDD and DMCD).

Table 1
The stability constant (*Ks* and log *Ks*) and Gibbs free energy change ($-\Delta G^0$) for the inclusion complexation of hosts with PPT guest in buffer solution (KH_2PO_4/K_2HPO_4, pH 7.0, 25 °C).

Sample	*Ks* (M^{-1})	log *Ks*	$-\Delta G^0$ (KJ·mol^{-1})
SACDD-CBD	3.353×10^6	6.525	37.25
TPACDD-CBD	7.540×10^5	5.877	33.55
DMCD-CBD	3.279×10^5	5.516	31.49
α-CD-CBD [12]	1.000×10^3	3.000	17.13
β-CD-CBD [12]	3.000×10^2	2.477	5.71
γ-CD-CBD [12]	1.500×10^3	3.176	18.13

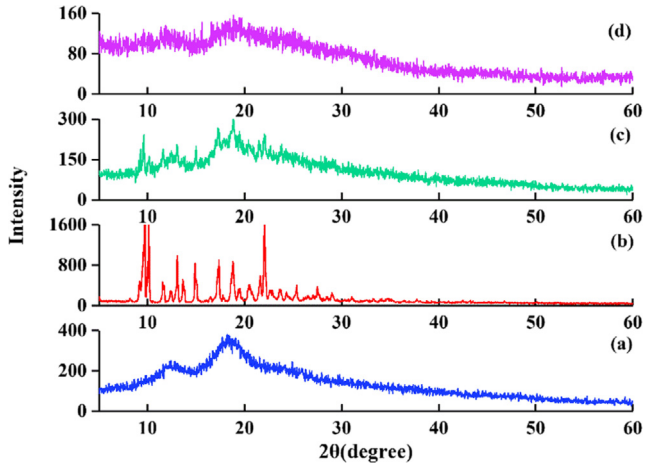

Fig. 3. Powder XRD patterns: (a) SACDD; (b) CBD; (c) SACDD-CBD physical mixture; (d) SACDD-CBD inclusion complex.

3.3. SEM analysis

SEM analysis is ideal tool for visualizing the surface morphology of the particles, SEM microphotographs of SACDD CBD, their physical mixture and inclusion complexes are displayed in Fig. 4. SEM image reveals irregular shaped plate like structure of SACDD

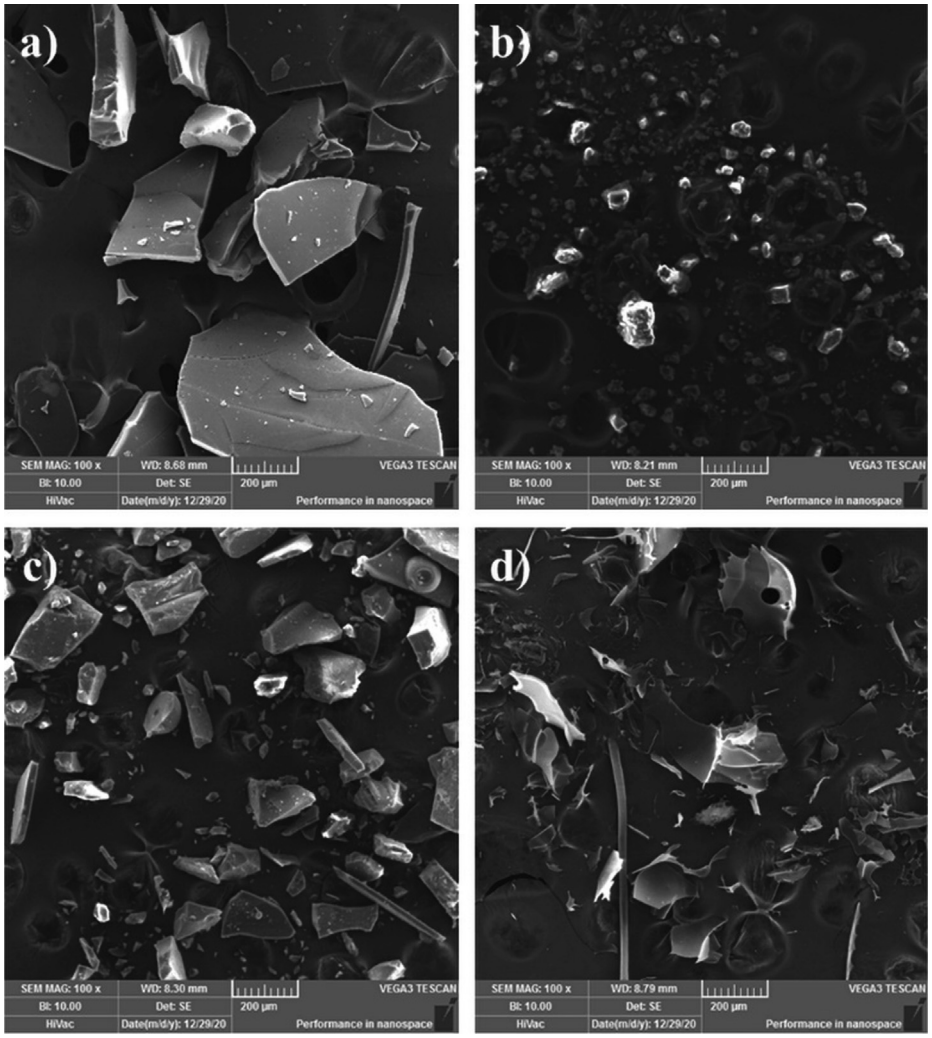

Fig. 4. SEM microphotographs: a) SACDD; b) CBD; c) SACDD-CBD physical mixture; d) SACDD-CBD inclusion complex.

(Fig. 4a), while CBD appears as irregular crystal particles (Fig. 4b). SEM image of SACDD-CBD physical mixture shows a mixture of irregular particles of CBD and block-like crystal of SACDD (Fig. 4c), it indicates CBD just adhered to SACDD surface. In contrast, the irregular crystal particles of CBD disappeared, while distinct amorphous structure with lesser dimensions were observed due to the formation of inclusion of complex (Fig. 4d). These morphological changes confirmed the formation of inclusion complex. The morphological changes in the SEM images of TPACDD-CBD (Fig. S10) and DMCD-CBD (Fig. S11) also confirmed they formed inclusion complexes.

3.4. ^1H and 2D ROESY NMR spectra analysis

When the guest molecule is incorporated into CD's cavity, the CD's protons inside the cavity (H-3 and H-5) should be sensitive to the changed chemical environment, and this would result in chemical shift changes of protons [39]. ^1H NMR spectra of CBD, SACDD and SACDD-CBD inclusion complex are shown in Fig. 5. The variations of ^1H NMR chemical shifts of SACDD before and after the formation of inclusion complex with CBD were listed in Table 2. Due to the insolubility of CBD, its ^1H NMR spectrum can only be obtained in $CDCl_3$ (Fig. 5a). When the SACDD-CBD inclusion complex was formed, obvious proton peaks of CBD (H-b, H-e, j, g, H-s, t and H-u, et.ac) in D_2O can be observed, it indicates solubility

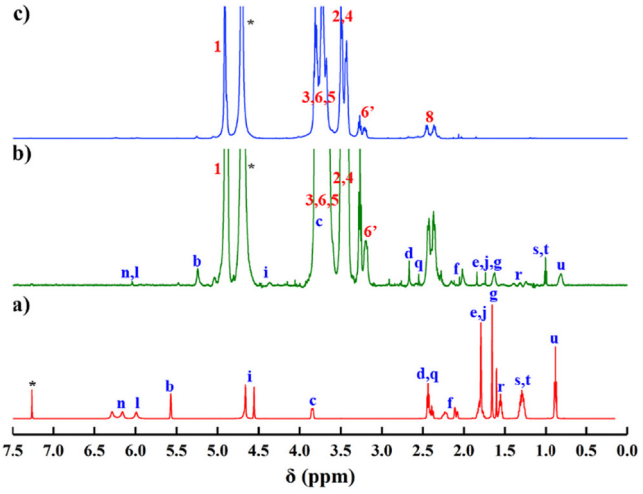

Fig. 5. ^1H NMR spectra: a) CBD in $CDCl_3$; b) SACDD-CBD inclusion complex in D_2O; d) SACDD in D_2O. "*" represents the solvent peak.

of CBD is remarkably increased. Besides, compared with SACDD (Fig. 5c), the H-3 and H-5 protons of SACDD in SACDD-CBD inclusion complex both underwent up field shifts of 0.04 and

Table 2
Chemical shifts of SACDD in free and complexed state determined in D_2O.

Protons	Chemical shift (ppm)		
	Δ_{SACDD}	$\delta_{complex}$	$\Delta\delta$ ($\delta_{complex} - \Delta_{SACDD}$)
H-1 of SACDD	4.99	4.96	−0.03
H-2 of SACDD	3.58	3.57	−0.01
H-3 of SACDD	3.90	3.86	−0.04
H-4 of SACDD	3.51	3.50	−0.01
H-5 of SACDD	3.76	3.68	−0.08
H-6 of SACDD	3.81	3.80	−0.01
H-6′ of SACDD	3.35	3.34	−0.01
H-8 of SACDD	2.49	2.47	−0.02

0.08 ppm (Fig. 5b). These phenomena indicate that CBD has been encapsulated in the cavity of SACDD. Similar results were obtained from the ^{1}H NMR spectra of TPACDD-CBD inclusion complex (Fig. S12, Table S1) and DMCD-CBD inclusion complex (Fig. S13, Table S2).

2D ROESY NMR experiments were used to further study the possible inclusion modes of CDs-CBD inclusion complexes. The 2D ROESY NMR spectrum for SACDD-CBD inclusion complex [40] was shown in Fig. 6. The spectrum revealed sophisticated NOE correlations between H-b, H-e, H-f protons of CBD (ring A) and inner cavity H-3, H-5 protons of SACDD respectively. Moreover, we could find cross-peaks between H-r, H-s, H-t, H-u protons in alkane side chain of CBD and inner cavity H-3, H-5 protons of SACDD. For the aromatic protons (ring B) of CBD, we can clearly see that the H-n, H-l protons of CBD were correlated to the H-5 proton of SACDD. Based on these results, as one CD's height is 0.79 nm, and CBD is typical long-chain molecular structure [41], we speculated CBD may enter two cyclodextrin cavities in bridged CDs simultaneously from the narrow side. The same inference was obtained from 2D ROESY (Fig. S14) of the inclusion complex of TPACDD and CBD. Based on these results, together with the 1:1 stoichiometry, we deduced the possible inclusion mode of SACDD with CBD as illustrated in Fig. 7a.

It is also worth noting that the inclusion mode of DMCD-CBD is different from SACDD-CBD and TPACDD-CBD. The 2D ROESY NMR spectrum for DMCD-CBD inclusion complex as is illustrated in Fig. S15. H-b, H-e and H-f protons of CBD were correlated to H-3 and H-5 protons of DMCD, while H-q, H-r, H-s, H-t and H-u protons of CBD were also correlated to H-3 and H-5 protons of DMCD. However, DMCD has only one cavity, we suggested that ring A of CBD enter the cavity of DMCD from narrow side, and alkane side chain of CBD penetrate into the other DMCD's cavity from narrow side, the possible inclusion mode of DMCD with CBD as illustrated in Fig. 7b.

3.5. Water solubility

We further measured the water solubility of three CDs-CBD inclusion complexes in Table 3. The aqueous solubility of natural CBD was reported to be 6.27×10^{-5} mg·mL^{-1} [42] After the complexation with CDs (SACDD, TPACDD and DMCD), the solubility of CBD significantly increased to 42.38, 28.34 and 36.57 mg·mL^{-1}, which are increased by 6.76×10^5, 4.52×10^5 and 5.83×10^5 folds, respectively. Lv et al. once used natural CDs (α-CD, β-CD and γ-CD), HP-β-CD-CBD and HP-γ-CD-CBD to increase the solubility of CBD (3.70, 2.10, 5.30, 4.90 and 0.90 mg·mL^{-1}), it's only increased by 5.90×10^4, 3.35×10^4, 8.45×10^4, 7.81×10^4 and 1.44×10^4 folds, respectively [12,13]. These results indicated that two bridged CDs (SACDD, TPACDD) and DMCD might be promising carriers to enhance the solubility of CBD.

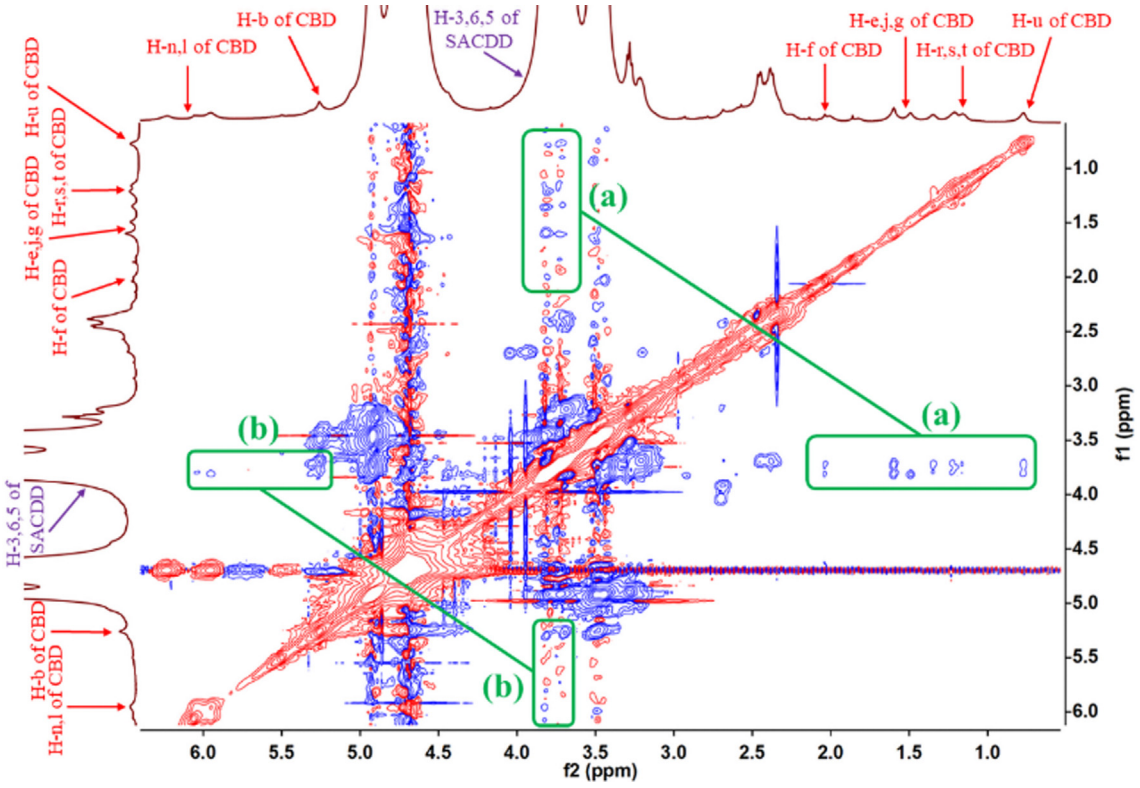

Fig. 6. 2D ROESY NMR spectrum for SACDD-CBD inclusion complex in D_2O: a) cross-peaks between H-f, H-e, H-r H-s, H-t, H-u protons of CBD and H-3, H-5 protons of SACDD; b) cross-peaks between H-n, H-l, H-b protons of CBD and H-3, H-5 protons of SACDD.

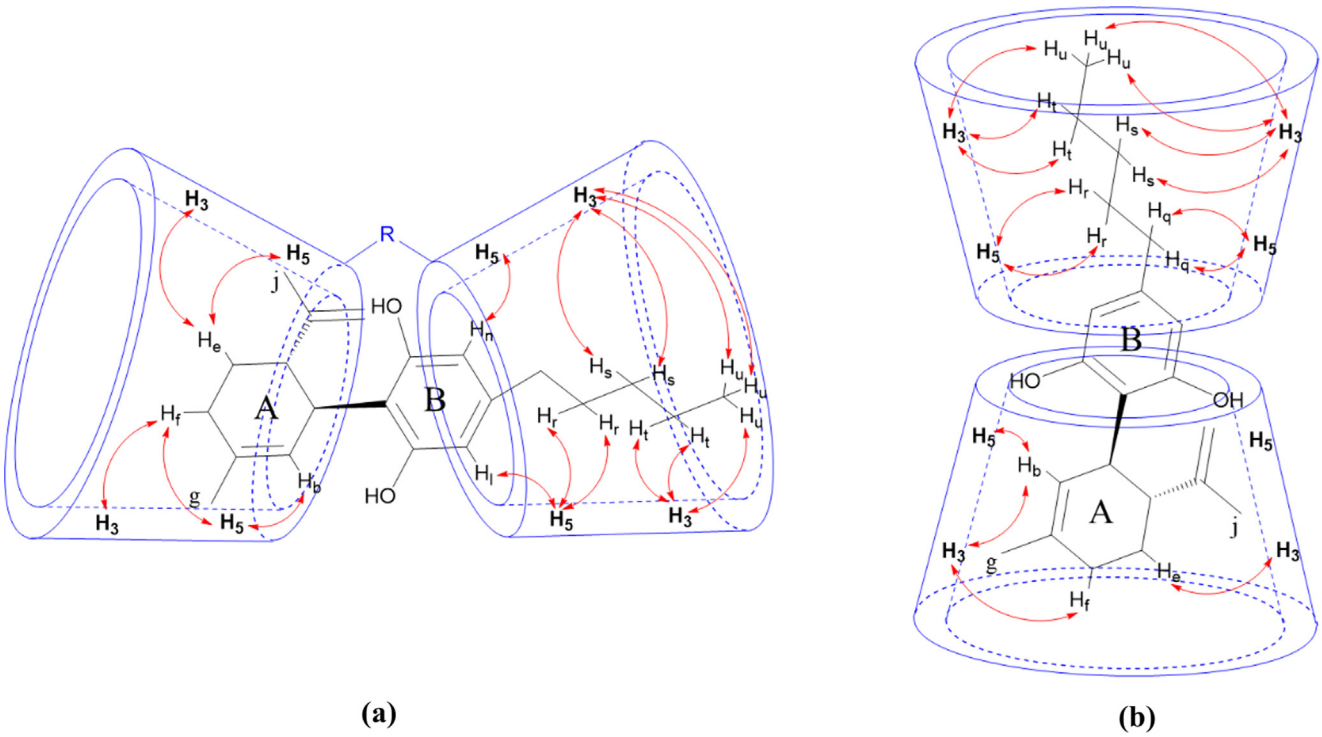

Fig. 7. Possible inclusion modes of CDs-CBD inclusion complexes: (a) SACDD-CBD inclusion complex (R = –NHCOCH$_2$CH$_2$CONH–) or TPACDD-CBD inclusion complex (R = –NHCOCH$_2$CH$_2$S CH$_2$CH$_2$CONH–); (b) DMCD-CBD inclusion complex.

Table 3
The water solubility of CBD and its inclusion complexes in buffer solution (KH_2PO_4/K_2HPO_4, pH 7.0, 25 ℃).

Sample	Water solubility of CBD (mg·mL^{-1})	Fold increase than free CBD
CBD [42]	6.27×10^{-5}	1.00
SACDD-CBD	42.38	6.76×10^5
TPACDD-CBD	28.34	4.52×10^5
α-CD-CBD [12]	3.70	5.90×10^4
β-CD-CBD [12]	2.10	3.35×10^4
γ-CD-CBD [12]	5.30	8.45×10^4
DMCD-CBD [13]	25.50	4.07×10^5
HP-β-CD-CBD [13]	4.90	7.81×10^4
HP-γ-CD-CBD [13]	0.90	1.44×10^4

3.6. Thermal analysis

In the field of supermolecule, TGA is often used to study thermal properties of complexes [43]. TG curves of SACDD, CBD, their physical mixture and inclusion complex was shown in Fig. 8. Two weight losses were observed in Curve a (SACDD's TG curve): the first weight loss was below 120 °C, which is caused by evaporation of water molecules; the second weight loss was above 300 °C, which is attributed to SACDD's degradation. Curve b shows that CBD had only one weight loss and completely degraded below 325 °C. The SACDD-CBD physical mixture (curve c) shows that a simple superposition of the CBD and SACDD curves. However, two weight losses were also observed in inclusion complex: the first weight loss was below 100 °C, which is caused by the water evaporation; the second weight loss was above 320 °C, it is due to inclusion complex's degradation. It is obvious that the thermal stability of CBD in inclusion complex was significantly enhanced. Analysis of TG curves of TPACDD-CBD (Fig. S16) and DMCD-CBD (Fig. S17) show similar results of SACDD-CBD discussed above.

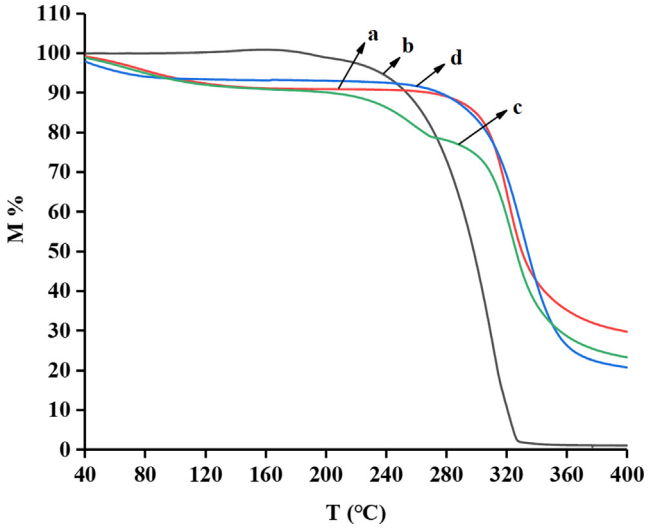

Fig. 8. TG curves of a) SACDD, b) CBD, c) SACDD-CBD physical mixture; d) SACDD-CBD inclusion complex.

3.7. In vitro antioxidant activity

The potential antioxidant activity of CBD has been reported, which is related with its neuroprotective activity [44,45]. Whether our CDs (DMCD, SACDD and TPACDD) could enhance the antioxidant capacity of CBD by formation of inclusion complexes was also investigated. The data of EC_{50} represents the antioxidant capacity of compound, it is the concentration of antioxidant required for 50% reduction of the radicals. The lower EC_{50} indicates the higher the antioxidant activity of a complexes [46,47]. As is shown in Fig. 9, all the CDs barely shows any antioxidant activity, and the EC_{50} value of inclusion complexes of CBD with different CDs was

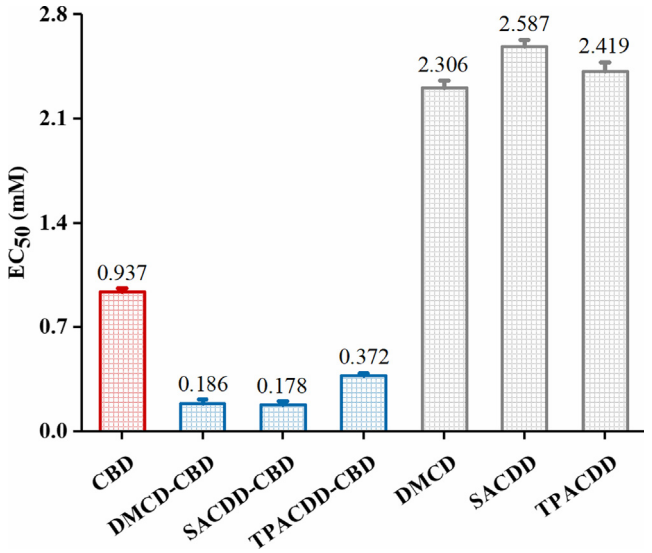

Fig. 9. In vitro antioxidant activity of inclusion complexes of CBD with CDs (CBD was dissolved in ethanol; CDs and three inclusion complexes were dissolved in water; EC_{50}, mM).

0.186 mM (DMCD), 0.178 mM (SACDD) and 0.372 mM (TPACDD) respectively, which is smaller than that of CBD (0.937 mM). It indicates that antioxidant activity of CBD was promoted effectively by forming inclusion complexes with CDs (DMCD, SACDD and TPACDD).

3.8. In vitro cytotoxicity

The cytotoxicity in vivo of compounds against cancer cell (MCF-7, HT29) and normal cell (HLF) were evaluated by MTT assay, and cisplatin was used as the positive control [48]. As is shown in Table 4, DMCD, SACD and TPACD barely show cytotoxicity against cells, which confirms their biosafety as CDs host carriers. Furthermore, all the inclusion complexes were less toxic than free CBD and they still maintained effective cytotoxicity against all the tested cancer cells, especially towards MCF-7 cell lines. Moreover, inclusion complexes reduced the toxicity of CBD to normal cell significantly. The results indicate that the cytotoxicity of the CBD-cyclodextrin complexes differs in cellular lines, it is might be induced by an enhancement of cellular membranes permeability, which was caused by an interaction between CDs and cholesterol contained in cancer cell membranes, while a less amount cholesterol was founded in normal cell membranes. Moreover, their interaction may induce a cellular endocytosis mechanism or a creation of transient channels in membranes, which may lead to a more effective cellular internalization of inclusion complexes in cancer cell rather than normal cell [49,50].

Table 4
Cytotoxic activities of CBD and inclusion complex in vitro (IC_{50}, μM).

	HLF	MCF-7	HT29
CBD	35.12	5.54	6.66
DMCD	>200	>50	>50
SACD	>200	>50	>50
TPACD	>200	>50	>50
CBD-DMCD	>200	33.46	35.24
CBD-SACD	>200	32.73	42.10
CBD-TPACD	>200	18.24	>50
Cisplatin	28.34	>50	20.00

4. Conclusion

In this work, we successfully designed and prepared two new bridged CDs dimers (SACDD and TPACDD) and their inclusion complexes with CBD. In addition, a reported DMCD-CBD inclusion complex was used as a comparison. Their characteristics and inclusion complexation behaviors have been investigated. After the complexation with SACDD and TPACDD, the water solubility of CBD was enhanced significantly by 6.76×10^5 and 4.52×10^5 folds, respectively. In vitro antioxidant activity of inclusion complexes of CBD was also improved, MTT assays shows their biosafety to normal cell as well as significantly enhanced anti-tumor activity. Our studies may provide a new strategy to improve water-solubility, biosafety and antioxidant activity of CBD in the field of pharmaceutical and commercial application.

CRediT authorship contribution statement

Liyuan Chen: Investigation. **Waixiang Yang:** Investigation, Writing - original draft. **Chuanzhu Gao:** Visualization. **Xiali Liao:** Supervision. **Jing Yang:** Investigation. **Bo Yang:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2021.117017.

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