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Enhancement of rhubarb extract solubility and bioactivity by 2-hydroxypropyl-β-cyclodextrin

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ABSTRACT

Rhubarb is a traditional Chinese medicinal herb, and the ethanolic extract of rhubarb consists of active anthraquinones, which are hydrophobic and have antiproliferative effects on hepatoma cell lines. To increase the aqueous solubility of rhubarb and study the consequent bioavailability, the ethanolic extract of rhubarb was complexed with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), a cyclic oligosaccharide that has a hydrophilic outer surface and a hydrophobic central cavity, to form a rhubarb–HP-β-CD complex. This complex was characterized by performing nuclear magnetic resonance spectroscopy, two-dimensional rotating frame spectroscopy and thin layer chromatography to confirm the inclusion of anthraquinones from rhubarb extract in HP-β-CD (weight ratio of rhubarb extract:HP-β-CD = 1:9). We investigated the effects of complexing rhubarb extract with HP-β-CD on the growth of Huh7 and HepG2 cells by performing cytotoxicity analysis, cellular uptake test, and colony formation assay. Our results showed that complexation of rhubarb extract with HP-β-CD increased the aqueous solubility and bioavailability of rhubarb and thus enhanced its effect on hepatoma cells.

1. Introduction

Rhubarb, a traditional Chinese medicinal (TCM) herb, consists of the root and rhizome of *Rheum palmatum* L. and is listed in the Chinese, Japanese, and European pharmacopoeias (Zhao, Wang, Zhou, Shan, & Xiao, 2009). Rhubarb is widely used as a laxative or a liver cleanser in the treatment of indigestion and jaundice (Fang et al., 2011; Fok, 2001; Tsai, Tsai, & Chang, 2004; Wang et al., 2011; Yu et al., 2005). The major components of the ethanolic extract of rhubarb include anthraquinone derivatives such as emodin, chrysophanol, rhein, aloe-emodin, and physicione and are shown in Fig. 1(a). These anthraquinones are hydrophobic and have a number of biological effects such as anticancer (Huang, Lu, Shen, Chung, & Ong, 2007; Shoemaker, Hamilton, Dairkee, Cohen, & Campbell, 2005), hepatoprotective (Arosio et al., 2000), antimicrobial (Céline et al., 2004), and anti-inflammatory effects (Tseng, Lee, Chen, Wu, & Wang, 2006). In particular, these anthraquinones have antiproliferative effects on hepatocellular carcinoma cells (Hsu et al., 2010; Shieh, Chen, Yen, Chiang, & Lin, 2004); hepatocellular carcinoma is the one of the cancers with most fatalities and is the most severe complication of chronic liver disease in the world (Parkin, Bray, Ferlay, & Pisani, 2005).

Naturally occurring cyclodextrins (CDs) are cyclic oligosaccharides consisting of (α-1,4)-linked-glucopyranose units. Each CD has a hydrophilic outer surface and a central hydrophobic cavity (Brewster & Loftsson, 2007; Del Valle, 2004; Uekama, Hirayama, & Irie, 1998). The most common CDs are α-, β-, and γ-CDs, which consist of 6, 7, and 8 glucopyranose units, respectively. The important characteristic of these CDs is their ability to act as host molecules or containers in the formation of inclusion compounds with many hydrophobic guest molecules in aqueous solution. Inclusion complexes of native and modified CDs with many species have been widely described in the fields of supramolecular chemistry (Li & Loh, 2008), pharmacology (Vyas, Saraf, & Saraf, 2008), food science (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara, 2009), and cosmetics (Cal & Centkowska, 2008).

Recently, instead of using a single compound as a guest molecule, crude extracts of plants such as cinnamon leaf (Ayala-Zavala et al., 2008), garlic oil (Ayala-Zavala et al., 2008), olive leaf (Mourtzinos, Salta, Yannakopoulou, Chiou, & Karathanos, 2007) and medical herbs (Feng et al., 2008; Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, & Makris, 2010; Michali, Karathanos, Chiou, & Mourtzinos, 2008), which contain multiple ingredients, are complexed with CDs to form an inclusion complex to improve the

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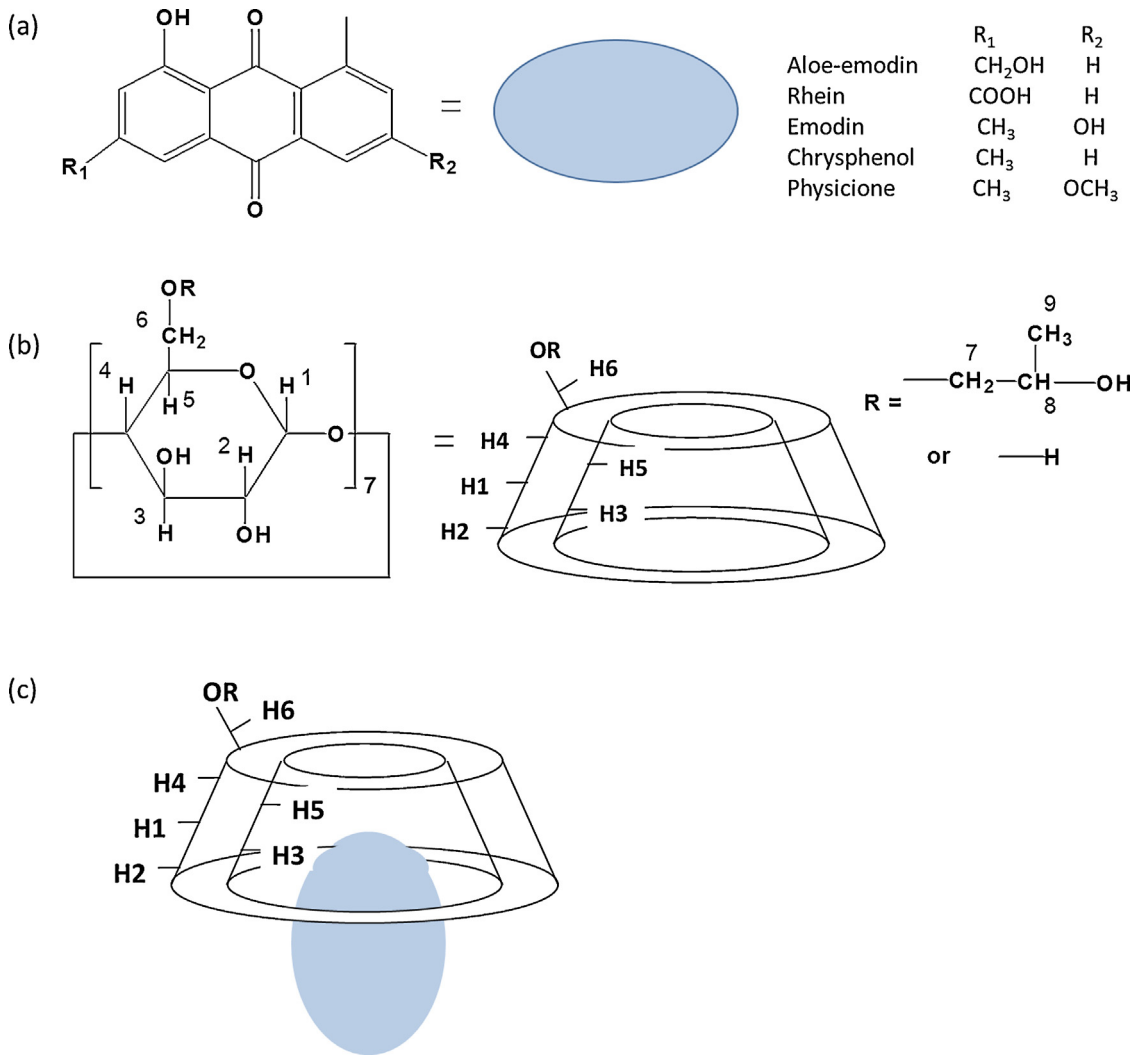

Fig. 1. (a) Chemical structure of anthraquinone derivatives, (b) 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), and (c) HP-β-CD inclusion complex.

thermal stability (Andreu-Sevilla, Carbonell-Barrachina, Lopez-Nicolas, & Garcia-Carmona, 2011; Michali et al., 2008; Mourtzinos et al., 2007), antioxidant capacity (Kalogeropoulos et al., 2010), antimicrobial activity (Ayala-Zavala et al., 2008), and anti-inflammatory activity (Feng et al., 2008) of these compounds.

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD), as presented in Fig. 1(b), is a hydroxyalkylated CD derivative and is widely used to increase the solubility, stability and bioavailability of drugs, because of its relatively higher water solubility and lower toxicity than β-CD (Gould & Scott, 2005). Since the active components of rhubarb, anthraquinones, are insoluble in water and limit the bioavailability of rhubarb, studies on the use of HP-β-CD to form an inclusion complex with the ethanolic extract of rhubarb and determination of the effect of this complex on inhibition of proliferation of human hepatoma cells are attracting attention. Furthermore, there are many solvents which can be used to extract the medical plants and influence the final ingredients of the extract. To the best of our knowledge, there is no standard condition for extraction. We used alcohol to extract rhubarb, because alcohol has been reported as the solvent for extraction of rhubarb for bioactivity study (Fang et al., 2011; He, He, Ma, & But, 2009; Ngoc et al., 2008; Tsai et al., 2004; Wang et al., 2011) and is less toxic than other organic solvents.

In this study, we prepared a complex of ethanolic extract of rhubarb and HP-β-CD using a freeze-drying method. Nuclear magnetic resonance (NMR) spectroscopy, two-dimensional rotating frame spectroscopy (2D ROESY) and thin layer chromatography (TLC) were used to ensure the inclusion of rhubarb extract with HP-β-CD. We examined the effects of complexing the rhubarb extract with HP-β-CD on the growth of hepatoma cell lines Huh7 and HepG2 by using cytotoxicity analysis, cellular uptake test, and colony formation assay.

2. Experimental

2.1. Materials

HP-β-CD (an average Mw ~1460 with 0.8 molar substitution, hydroxypropyl moiety) and phosphate buffered saline (PBS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Analytical grade ethanol and TLC plate (DC-Alufolien, Silicagel 60 F254, 0.2-mm thickness) were purchased from Merck (Darmstadt, Germany). (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) cell proliferation assay kit was obtained from Promega (Wisconsin, USA). Human hepatoma cell lines Huh7 and HepG2 were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2 and were subcultured every 3–4 days.

2.2. Preparation of crude extract of rhubarb

The air-dried slices of rhubarb, purchased from a Chinese medicine store (Taichung, Taiwan), were authenticated by pharmacognosists of China Medical University Hospital and were stored at 4 °C. In detail, we grinded 100 g of rhubarb and extracted it using 500 mL of 95% ethanol and stirred it at 25 °C for 24 h. The infusion was filtered to remove insoluble materials and freeze dried at −50 °C. The average yield of the rhubarb extract was approximately 11% (w:w). The dried extract was stored at −20 °C and was used in all experiments.

2.3. Preparation of rhubarb–HP-β-CD complex

We included rhubarb extract with HP-β-CD by various weight ratios (rhubarb extract:HP-β-CD = 1:1, 1:4 and 1:9). The solubility test showed the complex at the weight ratio of rhubarb extract:HP-β-CD = 1:9 had the best solubility. So the rhubarb–HP-β-CD complex at this ratio was used for consequent experiments. The preparation of rhubarb–HP-β-CD complex with different weight ratios by a freeze-drying method is described below. Various amount of HP-β-CD (2.7 g, 1.2 g or 0.3 g) were dissolved in 5 mL of water. We slowly added the ethanolic rhubarb extract, 0.3 g rhubarb in 30 mL of 95% ethanol, to the HP-β-CD solution with continuous agitation. The mixture was stirred continuously for 24 h at room temperature and was lyophilized in a freeze dryer at −50 °C to produce the rhubarb–HP-β-CD complex.

2.4. Solubility test

6 mg of rhubarb extract and 60 mg of rhubarb–HP-β-CD dispersed in 1 mL of water at 25 °C at 100 rpm in an orbital shaker (SK-24, SHIN KWANG, TW), respectively. Then both samples were centrifuged at 12,000 rpm (Biofuge Pico Heraeus Instruments, Germany) to remove clumps of insoluble rhubarb extract. The clear supernatant solution containing soluble rhubarb extract or rhubarb–HP-β-CD was collected and used for the solubility estimations. The calibration cure was obtained by dissolving various amounts of rhubarb extract in DMSO under identical conditions (Yallapu, Jaggi, & Chauhan, 2010). The rhubarb concentrations in the supernatant solution were determined using a NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) at the fixed wavelengths of 280 and 420 nm (Komatsu et al., 2006; Matsuda et al., 2001).

2.5. Thin layer chromatography

Sample solutions of HP-β-CD, rhubarb extract, and rhubarb–HP-β-CD complex were prepared by dissolving 0.09 g of HP-β-CD in 1 mL of water, 0.01 g of rhubarb extract in 1 mL of 95% ethanol, and 0.1 g of rhubarb–HP-β-CD complex in 1 mL of water, respectively. 0.01 g of rhubarb extract and 0.09 g of HP-β-CD dissolved in 1 mL of 95% ethanol was used as the solution of the physical mixture of rhubarb extract and HP-β-CD. Then, 1–4 μL of each sample solution was spotted on the silica gel TLC plate and developed in a mixed solvent (ethanol/ethyl acetate = 10/2, v/v). After development, the solvent was evaporated, and the dried plates were illuminated using an ultraviolet (UV) light to trace these samples. For sulfuric acid/heat treatment, the dried plates were immersed in an ethanol solution with 10% sulfuric acid followed by heating for 30 min at 60 °C to form charred blots.

2.6. ^{1}H NMR and 2D ROESY

^{1}H NMR spectra were obtained using a Bruker Avance 600NMR spectrometer using deuterated water (D_2O) or dimethyl sulfoxide (DMSO-d6) as the solvent. The chemical shifts (δ) were presented as ppm and referenced to the residual water signal (4.75 ppm).

2D ROESY was recorded on a Bruker-600 MHz instrument. The samples were prepared by dissolving 3 mg of the inclusion complex in about 1 mL of D_2O at room temperature. The spin-lock mixing time was set to 500 ms after calibration to ensure the reliability of ROESY cross-peaks.

2.7. MTS assay

Rhubarb extract and rhubarb–HP-β-CD complex were separately homogenized in DMEM and added to the media in doses equivalent to 100 and 150 μg/mL of rhubarb extract. Cell viability was measured 72 h after treatment with rhubarb extract and rhubarb–HP-β-CD complex and was assessed using an MTS cell proliferation assay kit in accordance with the manufacturer's instructions. All experiments were performed at least thrice.

2.8. Cellular uptake

The cellular uptakes of HP-β-CD, rhubarb extract, and rhubarb–HP-β-CD complex in Huh7 and HepG2 cells were analyzed by the fluorescence method. Briefly, cells (2×10^4/well) were incubated in a 12-well plate with HP-β-CD, rhubarb extract, or rhubarb–HP-β-CD complex for different time intervals. The concentration of HP-β-CD, rhubarb extract, and rhubarb–HP-β-CD complex in these cells was 1.35 mg/mL, 150 μg/mL, and 1.5 mg/mL, respectively, and the cells treated with rhubarb extract and rhubarb–HP-β-CD complex had doses equivalent to 150 μg/mL of rhubarb extract. Cells were washed twice with PBS to remove the added HP-β-CD, rhubarb extract, or rhubarb–HP-β-CD complex before examination under Olympus microscopy system CKX 41 (Olympus, Tokyo, Japan), and images were captured using a DP Controller and DP Manager software (Olympus). The emission spectra were recorded from 450 to 700 nm with an excitation wavelength of 525 nm for rhubarb extract and rhubarb–HP-β-CD complex. Average fluorescence intensity per cell was determined using IPLab Spectrum version 4.0.8 software (BD Biosciences, San Jose, CA).

2.9. Colony formation assay

Human hepatoma cell lines, Huh7 and HepG2, were seeded in 2 mL media in 6-well plates (2×10^4/well) and were incubated 24 h to initiate formation of colonies. Then, cells were treated with an equivalent dose of rhubarb extract 150 μg/mL over a period of 7 days. The plates were washed thrice with PBS, fixed in chilled methanol, stained with 0.5% crystal violet, washed with water, and air dried. The number of colonies was counted using ImageJ (National Institutes of Health, Bethesda, MD) software. The percentage of colonies formed was calculated using the number of colonies formed in treatment divided by the number of colonies formed in DMEM without drug.

3. Results and discussion

3.1. TLC analysis

TLC is a chromatographic technique used to separate mixtures. Different compounds in the sample mixture travel at different traveling rates because of the differences in their attraction to the stationary phase and their solubility in the solvent. Separation of components can be adjusted by changing the solvent. The TLC results of HP-β-CD, rhubarb–HP-β-CD complex, rhubarb extract and the physical mixture of rhubarb extract and HP-β-CD visualized under a UV light and sulfuric acid/heat treatment are shown in Fig. 2(a) and (b).

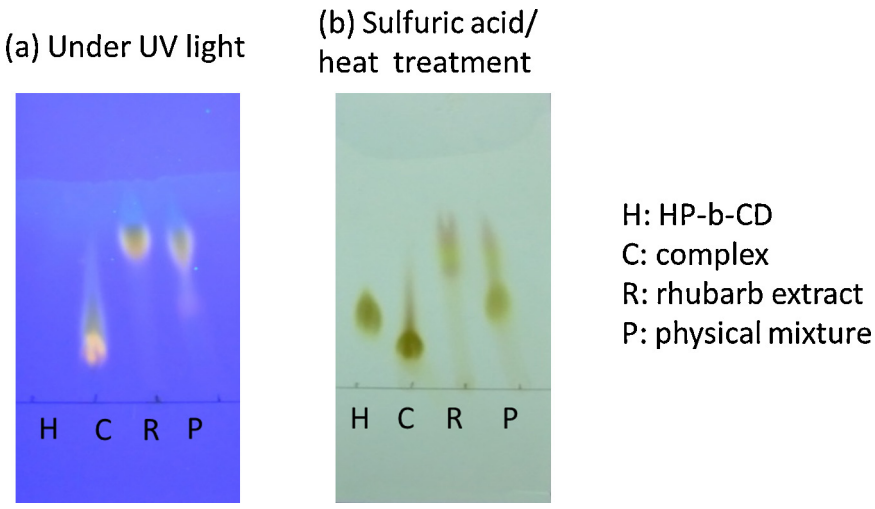

Fig. 2. Thin layer chromatography results of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), rhubarb–HP-β-CD complex, rhubarb extract and the physical mixture (a) under an ultraviolet light and (b) after treatment with sulfuric acid and heat.

Because HP-β-CD does not have a fluorescent moiety, no spot was observed under a UV light as shown in Fig. 2(a). However, the rhubarb–HP-β-CD complex, rhubarb extract and the physical mixture of rhubarb extract and HP-β-CD showed fluorescent spots which were from rhubarb. The rhubarb extract and the physical mixture traveled at a similar rate which was faster than the rhubarb–HP-β-CD complex. The similar traveling rates of rhubarb extract and the physical mixture suggested that rhubarb extract of the physical mixture was not encapsulated by HP-β-CD. The difference in the traveling rates between rhubarb extract and rhubarb–HP-β-CD complex indicated that rhubarb extract had been encapsulated in the CD cavity, and the interaction of rhubarb extract inside rhubarb–HP-β-CD complex with the stationary phase and developing solvent is different from that of rhubarb extract without inclusion.

HP-β-CD became visible after sulfuric acid/heat treatment to form charred blots as shown in Fig. 2(b). The physical mixture showed 2 spots: one for HP-β-CD and the other for rhubarb extract. Because HP-β-CD had the higher molecular weight, it showed a lower traveling rate than rhubarb extract. Furthermore, rhubarb–HP-β-CD complex had 2 spots, too. These two spots were generated by the breakdown of the rhubarb–HP-β-CD complex by the mixed solvent during developing process. HP-β-CD of the complex showed a lower traveling rate than pure HP-β-CD and the physical mixture, indicating rhubarb extract of the complex was included by HP-β-CD and slowed down its traveling rate by weight increase. Rhubarb extract from the complex also had a lower traveling rate than that of rhubarb extract without inclusion, which was similar to the TLC findings under a UV light.

3.2. ^{1}H NMR spectroscopic analysis

^{1}H NMR spectroscopic analysis is one of the most useful techniques to determine the chemical composition of organic compounds, including CD and its inclusion complexes. The ^{1}H NMR spectra of HP-β-CD are shown in Fig. 3(a). The proton resonances of HP-β-CD were assigned on the basis of findings from previous studies (Axel Castelli et al., 2008; Rezende et al., 2009; Tsao

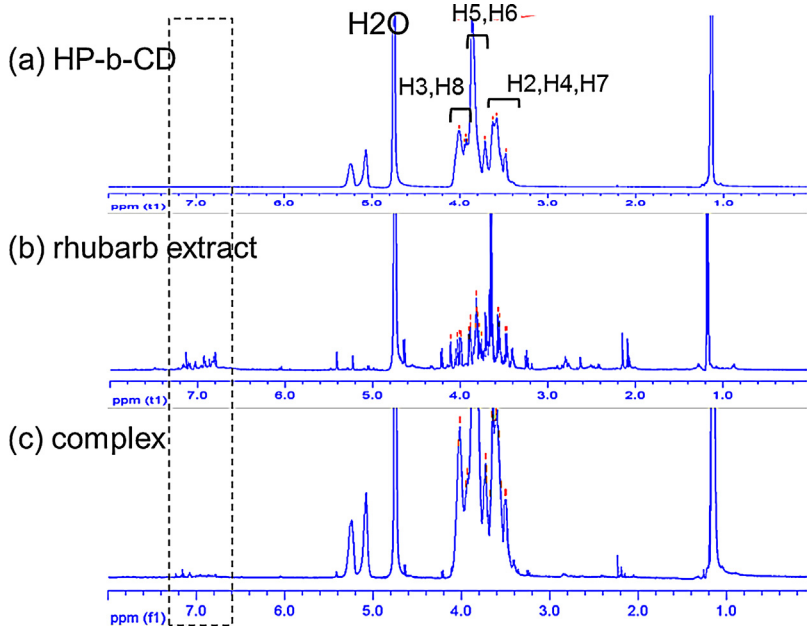

Fig. 3. Nuclear magnetic resonance (^{1}H NMR) spectra of (a) 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), (b) rhubarb extract, and (c) rhubarb–HP-β-CD complex.

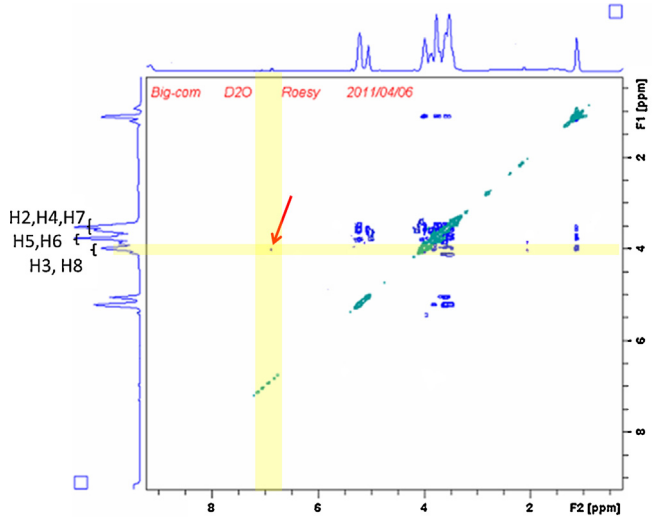

Fig. 4. Two-dimensional rotating frame spectroscopy (2D ROESY) of rhubarb–HP-β-CD complex.

et al., 2012). Chemical shifts of HP-β-CD, as shown in Fig. 1(b), in the range 3.85–4.10 ppm were those of H3 and H8, those in the range 3.50–3.75 ppm were those of H2, H4, and H7, and those in the range 3.70–3.85 ppm were those of H5 and H6. The ^{1}H NMR spectrum of rhubarb extract is shown in Fig. 3(b). Although the rhubarb extract was a mixture of many ingredients and the composition analysis for the specific individual element was not applied in this report, the appearance of chemical shifts in the 6–8 ppm range indicated that the rhubarb extract contained components with aromatic moieties, which are parts of the structure of the anthraquinone shown in Fig. 1(a) and have been reported (Wang, Wang, Yang, Luo, & Kong, 2013). The ^{1}H NMR spectrum of the rhubarb–HP-β-CD complex is shown in Fig. 3(c). Comparison of ^{1}H NMR spectra of rhubarb–HP-β-CD complex (Fig. 3c) with that of HP-β-CD and rhubarb extract (Fig. 3a and b) indicated that the rhubarb–HP-β-CD complex consisted of both the rhubarb extract and HP-β-CD.

3.3. 2D ROESY

2D ROESY is one of the most effective tools for investigating inter- and intra-molecular interactions of guest molecule–CD complex systems. The presence of cross-peaks, which are generated by nuclear Overhauser effects (NOE), between protons of guest molecules and CDs provides useful information about the dynamics and averaged relative inter-/intra-molecular proton distances of these species within 0.4 nm in solution (Ali & Upadhyay, 2008; Jullian, Moyano, Yanez, & Olea-Azar, 2007; Slavetinska, Mosinger, Dracinsky, & Posta, 2008; Tsai, Tsai, Wu, & Tsai, 2010).

Fig. 4 presents 2D ROESY spectrum of the rhubarb–HP-β-CD complex. No cross-peak was found between the aromatic protons (6–8 ppm) of rhubarb extract (anthraquinones) and H2/H4/H7 (3.50–3.75 ppm), the external protons of HP-β-CD, indicating rhubarb extract did not have any interaction with the outer surface of HP-β-CD.

However, a cross-peak between these aromatic protons and H3/H8 (3.85–4.10) was observed. This cross-peak was not caused by H8, the external protons of HP-β-CD, since anthraquinones of rhubarb extract showed no interaction with the outer surface of HP-β-CD. These results suggested that this cross-peak was generated by the interaction between H3, the internal proton of the wider rim sides of HP-β-CD, and rhubarb extract. Furthermore, no cross-peak was determined between these aromatic protons and H5/H6 (3.70–3.85 ppm), the internal proton of the narrower rim sides of HP-β-CD. These results suggested that anthraquinones of rhubarb extract partially entered into the cavity of HP-β-CD through the wider rim sides of HP-β-CD, as shown in Fig. 1(c).

Because of the partial inclusion of anthraquinone of rhubarb extract with HP-β-CD, rhubarb–HP-β-CD complex was broken down to rhubarb extract and HP-β-CD by the mixed solvent during developing process in TLC test.

3.4. Solubility

Since the rhubarb extract was a mixture, its UV spectrum was a combination of all compounds (not for a specific compound) and showed two absorption peaks around 280 and 420 nm, which were suggestive of anthraquinones (Komatsu et al., 2006; Matsuda et al., 2001). Therefore, the apparent maximal solubilities of

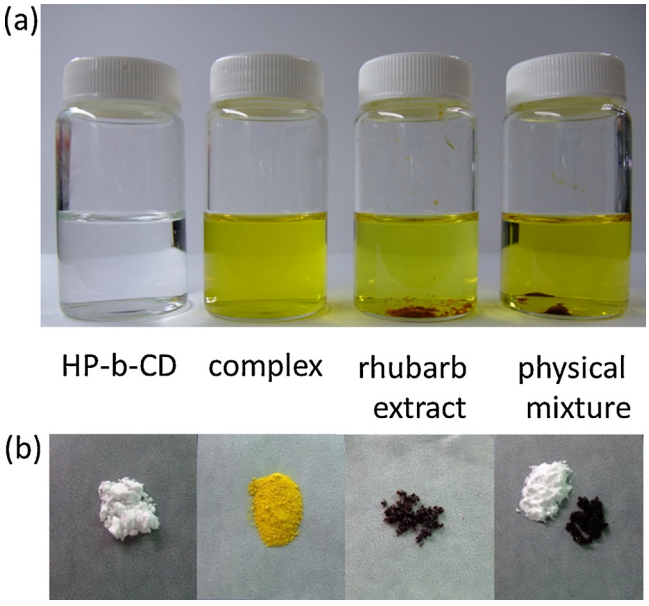

Fig. 5. Photographs of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), rhubarb–HP-β-CD complex, rhubarb extract and the physical mixture in (a) solution state and (b) solid state.

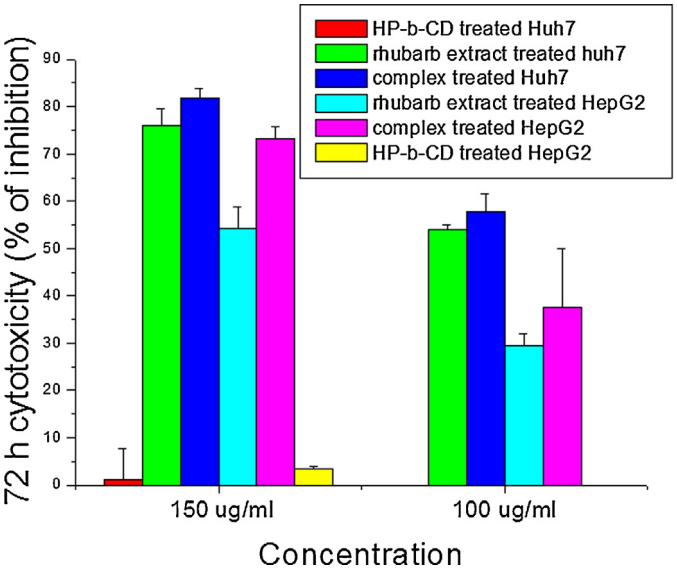

Fig. 6. Cytotoxicity of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), rhubarb extract, and rhubarb–HP-β-CD complex in Huh7 and HepG2 cells treated for 72 h.

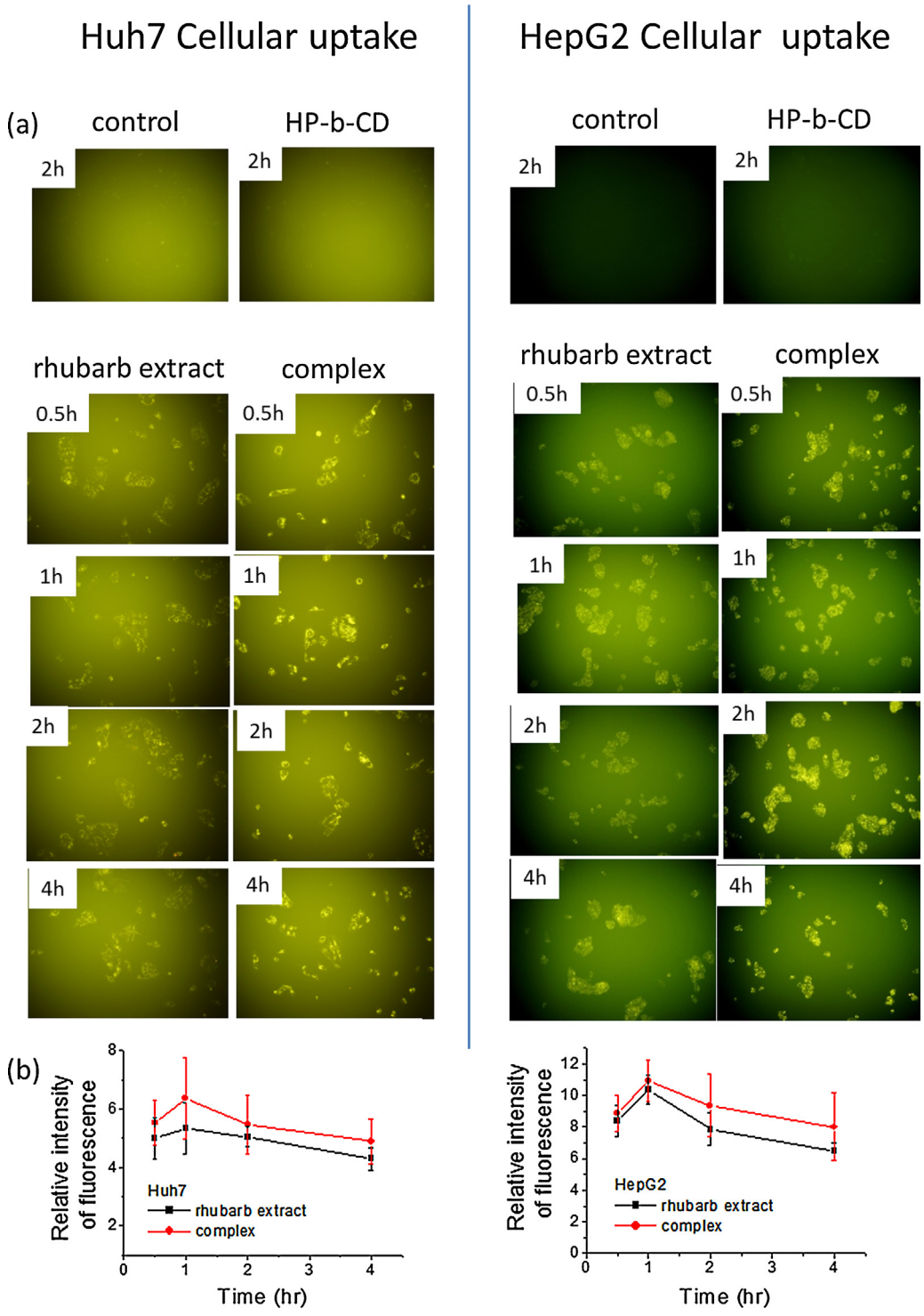

Fig. 7. (a) Fluorescent images and (b) relative fluorescent intensities of rhubarb uptake in Huh7 and HepG2 cells at different time intervals.

rhubarb were estimated at both wavelengths. The maximal rhubarb solubilities of rhubarb extract and rhubarb–HP-β-CD complex were 1.24 (at 280 nm)/1.12 (at 420 nm) mg/mL and 5.68 (at 280 nm)/5.56 (at 420 nm) mg/mL, respectively, indicating rhubarb–HP-β-CD complex had a higher solubility than rhubarb extract. The images of rhubarb–HP-β-CD, HP-β-CD, and rhubarb extract and the mixture of rhubarb extract and HP-β-CD in solution and in solid state are shown in Fig. 5(a) and (b), respectively. Rhubarb–HP-β-CD complex (100 mg in 2 mL water) and HP-β-CD (90 mg in 2 mL water) were water soluble, but rhubarb extract (10 mg in 2 mL water) and the mixture (10 mg of rhubarb extract and 90 mg of HP-β-CD in 2 mL water) were partially soluble in water, which indicated that the insoluble components of rhubarb extract had been included by HP-β-CD and thus its aqueous solubility was improved.

3.5. MTS assay

The MTS assay is a colorimetric assay for measuring the activity of enzymes that reduce MTS to formazan dyes and impart a purple color to the solution (an absorbance maximum at 490–500 nm).

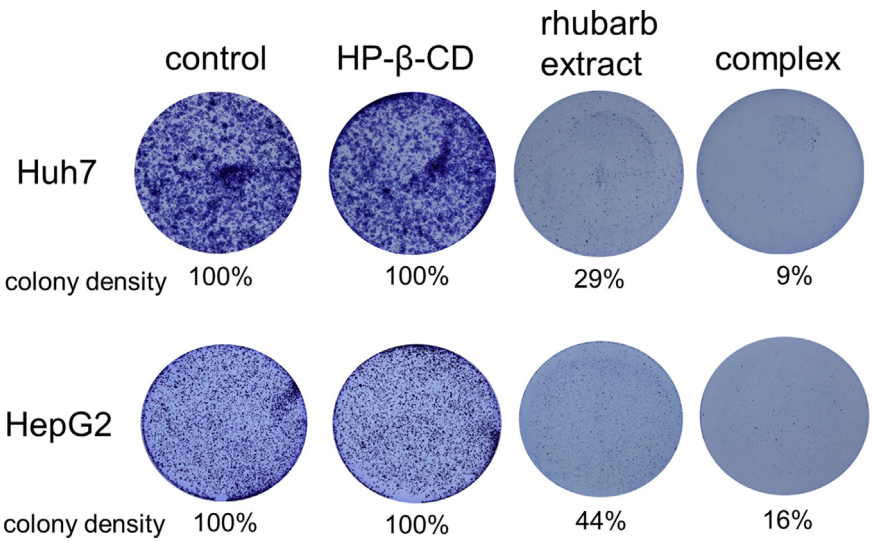

Fig. 8. Photographs of colony formation assay and colony densities of Huh7 and HepG2 treated with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), rhubarb, and rhubarb–HP-β-CD complex for 7 days.

In addition, this assay can be used to determine the cytotoxicity of potential medicinal agents and toxic materials because these agents would stimulate or inhibit cell viability and growth (Hsu et al., 2010). Therefore, we used MTS assay to evaluate the effects of the rhubarb extract and the rhubarb–HP-β-CD complex on the growth of hepatoma cell lines Huh7 and HepG2 for 72 h. The experimental results of MTS assay showed that IC50s for Huh7 cell of rhubarb and rhubarb–HP-β-CD were 91 and 84 μg/mL, respectively, and IC50s for HepG2 cell of rhubarb and rhubarb–HP-β-CD were 141 and 117 μg/mL, respectively. The cytotoxicity results at doses equivalent to 100 and 150 μg/mL of rhubarb extract and HP-β-CD dose of 150 μg/mL are shown in Fig. 6. The results showed that HP-β-CD had no cytotoxic effect on Huh7 or HepG2 cells. However, rhubarb extract and rhubarb–HP-β-CD complex inhibited the cellular growth in a dose-dependent manner. These results were consistent with those reported in previous studies in which the anthraquinone derivatives of rhubarb had antiproliferative effects of hepatocellular carcinoma cell lines (Hsu et al., 2010; Shieh et al., 2004).

The cytotoxic effect of the rhubarb–HP-β-CD complex on both Huh7 and HepG2 cells was greater than that of the rhubarb extract at equivalent doses of rhubarb extract. The increased cytotoxicity associated with the complex suggested a more efficient cellular uptake of rhubarb molecules from the rhubarb–HP-β-CD complex than from the rhubarb extract. This finding was related to the enhanced aqueous solubility of rhubarb molecules when they were complexed with HP-β-CD (Brewster & Loftsson, 2007). Other hydrophobic drugs, such as curcumin (Yallapu et al., 2010), dichloro(dipyridine)platinum (Horvath et al., 2008), and pyrazolopyrimidines (Botta et al., 2010), have also shown enhanced cellular uptake when complexed with CDs and thus enhancing their cytotoxic effect.

3.6. Cellular uptake

Rhubarb extract has an inherent fluorescence property, which we have utilized in the cellular uptake studies (Yallapu et al., 2010). The results of rhubarb uptake in Huh7 and HepG2 cells at different time intervals using fluorescence microscopy at doses equivalent to 150 μg/mL of rhubarb extract are shown in Fig. 7(a). The microscopic images of control cells and cells incubated with HP-β-CD did not show any fluorescence because the fluorescent rhubarb was absent in these cells. However, the cells treated with the rhubarb–HP-β-CD complex showed relatively greater fluorescence than the cells treated with rhubarb extract at the same time interval. These data suggest that HP-β-CD enhanced rhubarb uptake in the both cells, which was consistent with the results of cytotoxicity analysis.

Furthermore, average fluorescence intensity at different time intervals per cell was determined using IPLab Spectrum version 4.0.8 software (Yallapu et al., 2010). The results of the fluorescence kinetics are shown in Fig. 7(b). Both cells treated with the rhubarb–HP-β-CD complex had higher values of fluorescence intensity than those treated with the rhubarb extract. The maximum cellular uptakes of rhubarb were reached at about 1 h for all samples.

3.7. Colony formation assay

Colony formation assay is used to assess the ability of a cell to form a colony in soft agar. Previous studies suggest that colony formation assay provides the ability to evaluate long-term anticancer efficacy of the developed drug(s) or drug(s) formulations (Yallapu et al., 2011). The photographs of colony formation assays and quantitation of colony densities of Huh7 and HepG2 cells treated with HP-β-CD, rhubarb extract, and the rhubarb–HP-β-CD complex at a dose equivalent to 150 μg/mL of rhubarb extract for 7 days are shown in Fig. 8. Compared to the control cells, the cells treated with HP-β-CD showed no significant decrease in the density of colonies, which indicated that HP-β-CD had no inhibitory effect. Furthermore, the cells treated with rhubarb–HP-β-CD complex had lower colony density than those treated with rhubarb extract, which suggests an improved therapeutic efficacy of the rhubarb–HP-β-CD complex because of an intracellular uptake effect, and this finding was consistent with that of MTS analysis.

4. Conclusions

In this study, the rhubarb–HP-β-CD complex was prepared from an ethanolic extract of rhubarb and HP-β-CD by freeze-drying. TLC, ^{1}H NMR spectroscopy, 2D ROESY results showed that insoluble anthraquinones of rhubarb extract was included in the HP-β-CD to form the rhubarb–HP-β-CD complex. The rhubarb–HP-β-CD complex had higher aqueous solubility than rhubarb extract, which improved the uptake of rhubarb extract in hepatoma (Huh7 and HepG2) cell lines, and thus, increased the cytotoxicity of

rhubarb and decreased colony density. These results indicated that complexation of rhubarb extract with HP-β-CD enhanced the effect of rhubarb on Huh7 and HepG2 cell lines by increasing its water solubility and bioavailability.

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