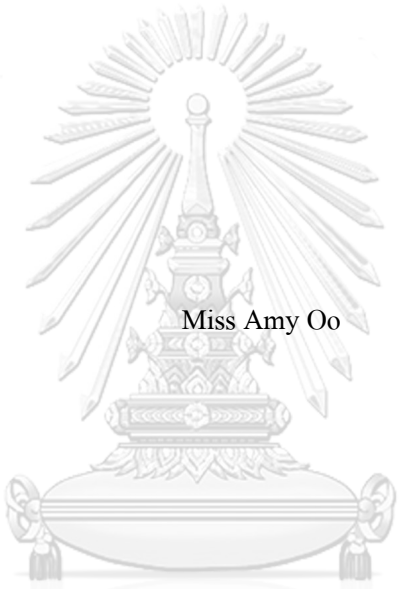
In silico AND i*n vitro* STUDIES ON INCLUSION COMPLEXATION OF ANTHRAQUINONE DERIVATIVES WITH BETA-CYCLODEXTRIN DERIVATIVES

Miss Amy Oo



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การศึกษาเชิงคอมพิวเตอร์และในหลอดทดลองของการเกิดสารประกอบเชิงซ้อนอินคลูชันของอนุพันธ์แอนทราควิโนนกับอนุพันธ์บีตาไซโคลเดกซ์ทริน

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Thesis Title	*In silico* AND i*n vitro* STUDIES ON INCLUSION COMPLEXATION OF ANTHRAQUINONE DERIVATIVES WITH BETA-CYCLODEXTRIN DERIVATIVES
By	Miss Amy Oo
Field of Study	Biochemistry and Molecular Biology
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เอมี่ อู :

การศึกษาเชิงคอมพิวเตอร์และในหลอดทดลองของการเกิดสารประกอบเชิงซ้อนอินคลูชันของอนุพันธ์แอนทราควิโนนกับอนุพันธ์บีตาไซโคลเดกซ์ทริน. (*In silico* AND i*n vitro* STUDIES ON INCLUSION COMPLEXATION OF ANTHRAQUINONE DERIVATIVES WITH BETA-CYCLODEXTRIN DERIVATIVES) อ.ที่ปรึกษาหลัก : ธัญญดา รุ่งโรจน์มงคล, อ.ที่ปรึกษาร่วม : ภาณุพงศ์ มหาลาภบุตรPh.D.

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Amy Oo : *In silico* AND i*n vitro* STUDIES ON INCLUSION COMPLEXATION OF ANTHRAQUINONE DERIVATIVES WITH BETA-CYCLODEXTRIN DERIVATIVES. Advisor: Assoc. Prof. THANYADA RUNGROTMONGKOL, Ph.D. Co-advisor: Panupong Mahalapbutr, Ph.D.

In this study, we investigated the cytotoxicity of 6 anthraquinones (ventilanone K, emodin, chrysophanol, aurantio-obtusin, 1-O-methyl-2-methyoxychrysophanol, and questin) toward A549 lung cancer cells and emodin (ED) exerted the most potent cytotoxicity with IC_{50} value of 34.27 ± 1.27 μM. The anti-cancer activity of ED has been well reported but its usage in practical applications has been restricted due to its poor solubility. To address this issue, we performed inclusion complexation of ED with βCD, hydroxypropyl-β-cyclodextrin (HPβCD), 2,6-di-O-methyl-β-cyclodextrin (DMβCD), and sulfobutylether-β-cyclodextrin (SBEβCD) theoretically using molecular dynamics simulations and experimentally using phase solubility study and cytotoxicity screening toward cholangiocarcinoma cell lines (KKU-213A and KKU-213B). The 500-ns MD simulations revealed that ED is able to form inclusion complexes with βCDs in two possible orientations: resorcinol ring insertion (R-form) and *m*-cresol ring insertion (C-form) mainly driven by van der Waals interaction. The ED in complex with SBEβCD (degree of substitution, DS-7) inclusion complex showed the highest number of atom contacts and the lowest solvent accessibility, in line with the ΔG_{bind} results ranked in the order of ED/SBEβCD (-6.18 ± 1.15 kcal/mol) > ED/HPβCD (-3.81 ± 0.65 kcal/mol) > ED/βCD (0.11 ± 0.51 kcal/mol). Consequently, the inclusion complexes between ED and βCDs (βCD, HPβCD, SBEβCD and DMβCD), were experimentally studied. The phase solubility testing provides AL-type diagrams indicating the 1:1 stoichiometry between ED and βCDs. Altogether, the stability constants of studied complexes can be ranked in the order of ED/DMβCD (1500 M^{-1}) > ED/HPβCD (1000 M^{-1}) > ED/βCD (980 M^{-1}) > SBEβCD with low degree of substitution (880 M^{-1}) at 25°C and the highest water solubility was observed in ED/DMβCD complex with 113.86 ug/mL. Moreover, ED/DMβCD complex shows the most promising inhibition with the IC_{50} values of 52.78 ± 1.57 and 47.00± 0.58 μM toward KKU-213A and KKU-213B, respectively.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
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Amy Oo

TABLE OF CONTENTS

LIST OF PUBLICATION

This thesis contains general summary (introduction, background information and conclusion) and the following manuscripts which are referred in the text by their roman number.

Manuscript I: Molecular encapsulation of emodin with various β-cyclodextrin derivatives: A computational study

Manuscript II: *In vitro* studies on inclusion complexation of emodin with various β-cyclodextrin derivatives

INTRODUCTION

1.1 Research concept

In this research, we aimed to enhance the water solubility and anti-cancer potential of anthraquinone variant, emodin (ED), by inclusion complexation with β-cyclodextrin (βCD) and its derivatives: hydroxypropyl-β-cyclodextrin (HPβCD), sulfobutylether-β-cyclodextrin (SBEβCD) and di-O-methyl-β-cyclodextrin (DMβCD). Briefly, six anthraquinone compounds extracted from *Ventilago harmandiana* Pierre namely ventilanone K, ED, chrysophanol, aurantio-obtusin, 1-O-methyl-2-methyoxychrysophanol, and questin were firstly tested for their cytotoxic potential toward A549 human lung cancer cell line. The most potent anthraquinone, ED, was selected for further studies. The molecular encapsulation of emodin with focused βCDs was theoretically studied by using all-atom molecular dynamics (MD) simulations and experimentally investigated by phase solubility testing and different characterization techniques. The enhanced anti-cancer potential of the prepared inclusion complexes compared to the unbound ED was studied on cholangiocarcinoma cell lines KKU-213A and KKU-213B.

1.2 Research rationality

On a global scale, cancer is responsible for an estimated 9.6 million of deaths as of 2018 and it represents the second most leading cause of death globally. Amongst different types of cancer, lung cancer is the most common and deadliest worldwide cancer and cholangiocarcinoma cancer is the leading cancer type in Thailand. The incidence report from Northeast part of Thailand accounted for ASR = 113.4/100,000 which was the highest incidence rate in the world. The current available options for cancer treatment include surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant and precision medicine. Out of

ample techniques, chemotherapy is widely used to treat various types of cancer. Despite its extensive usage, chemotherapy has numerous side effects including its empirical approach which is not only killing the rapidly growing cancer cells but also killing or slowing the growth of healthy cells. Another burden of chemotherapy application is that in addition to the significant expense of anti-cancer drugs itself, they are generally administered through intra-venous (IV) infusion in which patients should have been hospitalized making the treatment moderately costly and not-effectively accessible. Based on this ground, the usage of natural compounds and traditional herbs as an alternative feasible source of chemotherapeutic agents as well as oral medication system attributable to its simplicity of administration, have been gained much research attention in the development of new anti-cancer drugs.

The anthraquinone (anthracene 9,10 dione), a planar tricyclic benzene ring in structure, represents a biologically significant compound, ubiquitous in nature. A recent report described that Ventilago harmandiana Pierre, an ecologically important plant from southern part of Thailand, locally named as “Khon Tee Dum”, is also a rich source of anthraquinones including ED and pyranonaphthoquinones. It was historically used as dyes and a bird repellant. Following the discovery of anti-tumor drug mitoxantrone, the synthetic anthraquinone, which is the only US-FDA approved relapse-remitting treatment for multiple sclerosis, a large number of natural, semi-synthetic and synthetic anthraquinones have been evaluated for their wide array of pharmacological properties and have been utilized in the treatment of cancer, viral infection , bacterial infection and anti-inflammatory infection. Based on the structural modification with different functional groups, anthraquinone compounds show diverse mechanisms towards cancer cells. Some show anti-cancer activity by blocking DNA synthesis pathway in which both DNA topoisomerase II-alpha and beta are being blocked while some anthraquinones show inhibition activity on cell proliferation by

down-regulating DNA recombinase protein Rad51 and excision repair cross-complementary 1 (ERCC). Unfortunately, poor aqueous solubility and thus associated low bioavailability of anthraquinone compounds lead to a major obstacle to apply them in the development of new anti-cancer drugs in the economical and pragmatic way.

Various approaches to enhance drug solubility such as particle size reduction, salt formation, host-guest complexation, have been reported. It has been proven that encapsulation with cyclodextrin as drug carriers can potentially improve the solubility, bioavailability, pharmacological properties, and stability of many lipophilic guest molecules. Cyclodextrins, a group of cycloamyloses formed from the enzymatic digestion of starch by cyclodextrin-glycosyltransferase, represent a toroidal, hollow, truncated cone shape with narrow and wide rims, exposing its hydrophilic surface outside and arranging its hydrophobic cavity inside where the guest molecules are entrapped to form inclusion complex mainly driven by van der Waals interaction. The naturally occurring CDs are α-cyclodextrin (αCD), β-cyclodextrin (βCD), and γ-cyclodextrin (γCD), formed by α (1-4) glycosidic linkage between six, seven and eight D-glucopyranose units, respectively. Amongst the natural CDs, βCD has been widely used due to its feasible features such as perfect cavity size (6.0–6.5 Å diameter, 2.65 $Å^3$ volume), effective drug loading capabilities (guest molecule having molecular weights between 200 and 800 g/moL), high host-guest inclusion complex formation efficiency, commercial availability, and the relatively low cost. Despite its advantages, its intramolecular interactions (hydrogen-bond) among hydroxyl groups lead to the weaker interaction with the surrounding molecules resulting in lower solubility of βCD. To beat this boundary and to expand its usage in pharmaceutical applications, some functional groups are introduced to the primary and secondary hydroxyl groups of βCD. In recent years, hydroxypropyl-β-cyclodextrin (HPβCD), sulfobutylether-β-cyclodextrin (SBEβCD) and di-O-methyl-β-cyclodextrin (DMβCD) become the

commonly used derivatives because of their enhanced aqueous solubility and the lower incidence of βCD-related nephrotoxicity compared to the parent structure.

In this research, we firstly screened the anti-cancer potential of six anthraquinone compounds toward A549 cell line and the most potent ED was selected. The inclusion complexation of ED with βCDs was then studied with regards to enhance its water solubility and anti-cancer activity.

1.3 Research objectives

(i) To find the most potent candidate out of focused six anthraquinone compounds toward A549 cells

(ii) To enhance the water solubility and anti-cancer potential of the selected compound, ED, by inclusion complexation with studied βCDs

(iii) To propose the best host molecule for ED

1.4 Scope of the research

(i) To perform cytotoxicity screening of six anthraquinone compounds toward A549 cell line using MTT assay.

(ii) To perform the phase solubility study of the most potent compound, ED, with βCDs at different temperature settings to study the host-guest behavior.

(iii) To prepare the inclusion complexes between ED and βCDs by using freeze-drying methods.

(iv) To characterize the prepared inclusion complexes compared to the unbound forms and the physically mixed complexes by using scanning electron microscopy and differential scanning colorimetry.

(v) To explore the water solubility and anti-cancer potential enhancement of studied inclusion complexes.

1.5 Expected beneficial outcome(s) from the thesis

The success of this thesis could lead to the potential development of ED into pharmaceutical applications and the abundance of ED in “Khon Tee Dum” plant would provide an economical advantage for Thailand in the development of ED into commercial drug candidate.

CHAPTER I

MANUSCRIPT I

Molecular encapsulation of emodin with various β-cyclodextrin derivatives: A computational study

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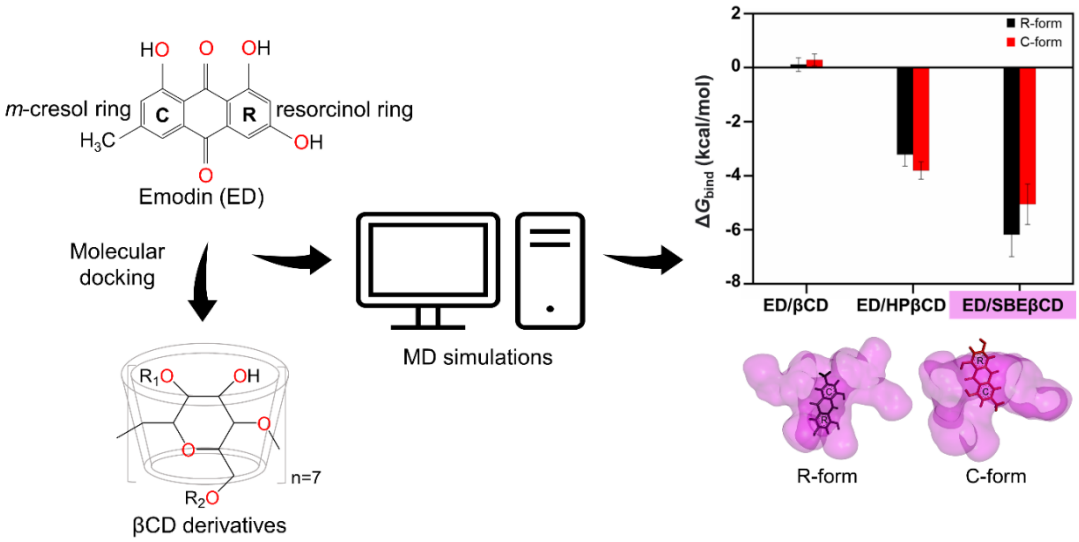
Abstract

Emodin (ED), one prominent variant of naturally occurring anthraquinones traditionally used in Chinese medicine, exhibits a wide spectrum of pharmacological properties. However, the poor aqueous solubility of ED limits its significant usage in practical applications. β-cyclodextrin (βCD) and its derivatives have been extensively utilized to enhance the water solubility and stability of lipophilic guest molecules by acting as a molecular shield through host-guest encapsulation. In this work, the structural dynamics details of inclusion complexation of ED with βCD and its two derivatives: 2-hydroxypropyl-β-cyclodextrin (HPβCD) and sulfobutylether-β-cyclodextrin (SBEβCD), were studied using all-atom molecular dynamics (MD) simulations and molecular mechanics/generalized Born surface area (MM/GBSA)-based binding free energy (ΔG_{bind}) calculations. The 500-ns MD simulations revealed that ED is able to form inclusion complexes with βCDs in two possible orientations: resorcinol ring insertion (R-form) and *m*-cresol ring insertion (C-form) mainly driven by van der Waals interaction. The ED/SBEβCD inclusion complex showed the highest number of atom contacts and the lowest solvent accessibility at the hydrophobic cavity, in line with the ΔG_{bind} results ranked in the order of ED/SBEβCD (−6.18 ± 1.15 kcal/mol) > ED/HPβCD (−3.81 ± 0.65 kcal/mol) > ED/βCD (0.11 ± 0.51 kcal/mol). All findings suggested that βCD derivatives, especially SBEβCD, could be the appropriate host for ED in the potential development of ED into pharmaceutical applications.

Keywords: Emodin, β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin, inclusion complex, molecular dynamics simulations.

Graphical abstract



1. Introduction

Emodin (ED), 6-methyl-1,3,8-trihydroxyanthraquinone (Fig. 1A), is a naturally occurring anthraquinone analog, mainly reported in three plant families: Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex* and *Polygonum* spp.) [1] and Rhamnaceae (*Rhamnus* and *Ventilago* spp.) [2]. A recent report described that *Ventilago harmandiana* Pierre, an ecologically important plant from southern part of Thailand, locally named as "Khon Tee Dum", is also a rich source of anthraquinones, including ED and pyranonaphthoquinones [3]. ED was reported to possess anti-breast cancer activity through inhibition of estrogen receptor α [4]. The DNA intercalation activity of ED was previously informed through the strong interaction between adenine and thymine base pairs and weak mediation between phosphate backbone of helical DNA [5]. ED was also stated to induce apoptosis through ROS-dependent mitochondrial signaling pathway in human lung adenocarcinoma cells [6]. The molecular inhibition mechanisms of ED, such as cell cycle arrest, inhibition of serine/threonine kinase, and suppression of tyrosine kinase, have also been well documented [7, 8]. Possessing peculiar biological potentials, ED draws research attention in recent years. However, in drug development, the pharmacokinetics parameters such as ADME (absorption, distribution, metabolism, and elimination) and physicochemical characteristics of a potential drug candidate also play an important role in practical applications [9]. Although ED possesses diverse biological activities, its pharmaceutical applications are limited by its low water solubility.

The enormous efforts have been intensively put onto studies to enhance the water solubility of poorly soluble drugs and drug-like substances, ranging from co-solvency, inclusion complexation with cyclodextrin to nano-engineered drug delivery system [10, 11]. The recent studies have proved that the water solubility of ED can be enhanced by inclusion complexation with β-cyclodextrin (βCD) [12] and 2-hydroxypropyl-β-cyclodextrin (HPβCD) [13]. Lately, encapsulation with βCDs

becomes an interesting alternative to improve the aqueous solubility of hydrophobic guest molecules due to its desirable price-performance status, preferred drug loading capacity, commercial availability, potential capability to enhance stability, dissolution rate, bioavailability and pharmacochemical properties of lipophilic guest molecules and its excellent biocompatibility [14, 15]. βCD, a natural compound produced from starch degradation by glucotransferase enzyme, consists of seven α-D-glucopyranose units connected by α-1,4 glycosidic linkage, possessing geometrically truncated-cone-shaped structure with relative hydrophobic inner cavity and hydrophilic outer surface. It usually hosts guest molecules through van der Waals interaction into its nanocavity [16]. Despite having favorable features, the low aqueous solubility (18.5 mg/mL at 25^{o}C) [17] and renal toxicity of βCD (0.9 g/kg in rats) [18] lead to the development of structurally modified derivatives such as HPβCD and sulfobutylether-β-cyclodextrin (SBEβCD) (Fig. 1B) [19-21]. Based on the fact that the solubility of SBEβCD (700 mg/mL) [22] is higher than that of the parent βCD, and the nephrotoxicity related to βCD does not exhibit in SBEβCD [23, 24]. Moreover, SBEβCD does not have cytotoxic effect on intestinal epithelial Caco-2 cells due to its minimal capacity to solubilize membrane lipids compared to methylated βCDs [25]. Accordingly, SBEβCD is considered as a safer drug carrier for oral administration [26].

Although the experimental studies on inclusion complexation of ED with βCD and HPβCD have been reported [12, 13], the structural details on those inclusion complexes as well as on the ED/SBEβCD complex remain largely unexplored. Therefore, in the present study, all-atom MD simulations and free energy calculations on inclusion complexation of ED toward βCD, HPβCD, and SBEβCD were performed to elucidate the structural dynamics details of the inclusion complexes in aqueous solution and to find the best host molecule for ED to enhance its water solubility,

which could be a supportive information in the employment of ED into pharmaceutical applications.

2. Computational details

2.1 System preparation

The 3D structure of ED was constructed and optimized by Gaussian09 program using HF/6-31G* level of theory [27]. The protonation state of ED was characterized using MarvinSketch software at pH 7.0. The HPβCD with low degree of substitution (DS) (4.55) exhibited a higher solubilizing capacity and a lower nephrotoxicity compared to HPβCD with medium DS (6.16) and high DS (7.76) reported by L. Pengyu *et al*. [28]. Moreover, our previous work reported that HPβCD with four HP-substitutions on the primary rim of βCD shows the lower probability of cavity self-closure by HP-substitutions [21]. Therefore, HPβCD with four HP-substitutions was selected for the present work and the 3D structures of βCD and HPβCD were taken from our previous studies [21, 29]. For SBEβCD, it was constructed by replacing the hydroxyl groups at O6 and O2 positions of native βCD with one to seven optimized sulfobutylether (SBE) groups (**Fig. S1**). As shown in **Fig. S2**, the potential energy surface (PES) profiles of SBEβCD with different DSs showed that SBEβCD with DS of 7, especially at the primary rim of βCD, could be the most suitable host by keeping its unique structure, a truncated cone shape, with the lower probability of flipping of glucopyranose units. Our results are agreement with the SBEβCD with an average DS of 6.5 as a novel parenterally safe solubilizer and stabilizer for poorly water-soluble drugs [23, 24, 30, 31]. Moreover, the previous study by Szabó,Z.I. *et al*. also showed that SBEβCD with DS of 7 on the primary rim of βCD is the best host for rasagiline inclusion complexation compared to the other forms of SBEβCD [32]. Based on these reports, SBEβCD with 7 SBE-substitutions on the primary rim of βCD was used in this study.

2.2 Molecular docking

The optimized ED and βCDs were used to generate inclusion complex by Accelrys Discovery Studio 2.5 (Accelrys Software Inc., San Diego, CA, USA) CDOCKER module. The preferential binding orientations of ED toward βCDs were studied using a setting of 10 Å as a docking sphere. The top 100 hits were then sorted out based on binding interaction score. After that, the percentage of docked conformation (%DCs) of the studied complexes was counted, and the representative structures with the lowest interaction energy were then selected as initial structures for MD simulations.

2.3 Molecular dynamics simulations

MD simulations of ED in complex with βCDs were performed using AMBER16 software package [33]. The partial charge and other necessary parameters were generated using standard procedures [21, 29, 34, 35]. The Glycam-06 [36] and general AMBER force fields (GAFF) [37] were applied on βCDs and ED, respectively. Using LEaP module, TIP3P water molecules were added to solvate each inclusion complex with a spacing distance of 15 Å. In the case of ED/SBEβCD system preparation, seven Na^+ ions were added to neutralize the system, since SBEβCD contains negatively charged sulfobutylether groups. The added water model was then minimized using 1500 steps of steepest descent (SD), followed by 3000 steps of conjugated gradient (CG). Subsequently, the whole system was minimized using the same procedure. Each solvated model was then heated up from 10 K to 298 K for 100 ps using a canonical ensemble (*NVT*) and equilibrated for another 1000 ps. Molecular dynamics (MD) simulation with isothermal-isobaric ensemble (*NPT*) was performed under periodic boundary condition at 1 atm and 298 K, with a time step of 2 fs. To fix all chemical bonds including hydrogen, the SHAKE algorithm [38] was applied. The

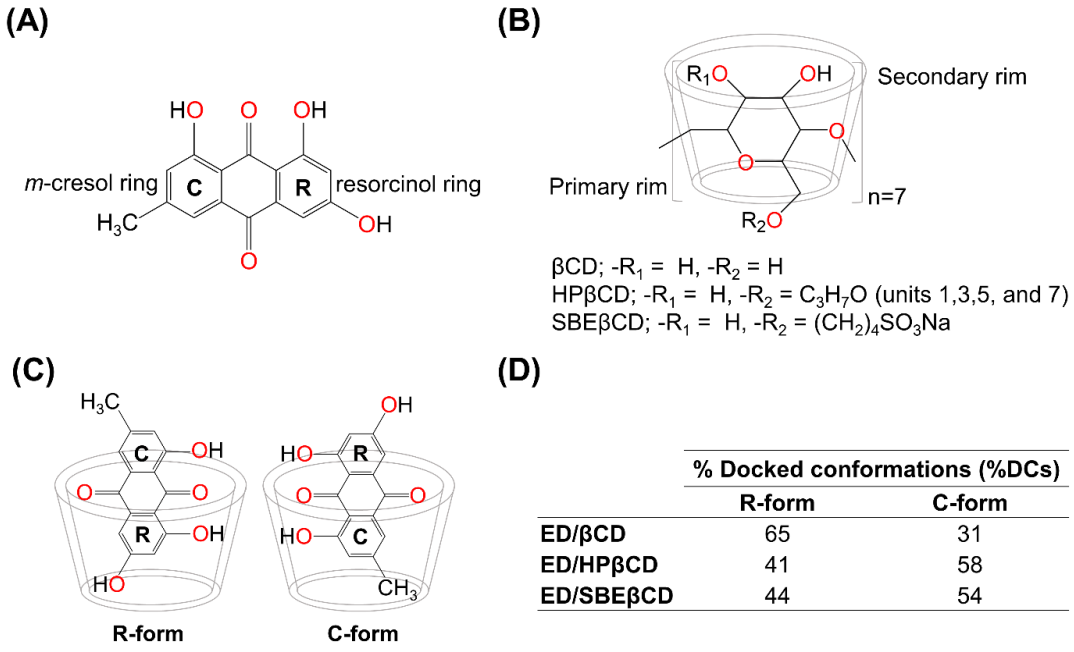
cutoff distance for non-bonded interactions was set to 12 Å, while the Particle Mesh Ewald [39] method was used to treat long-range electrostatic interactions. All-atom MD simulations of each system were performed for 500-ns in three replicates with the different initial velocities (MD#1-3). The system stability with equilibrium status was checked using root mean squared displacement (RMSD) and the last 100-ns (400-500ns) of each system were then selected for further analysis. After that, the preferred binding orientation of each system was then analyzed based on distance analysis and RMSD clustering was applied to explore the representative clusters of each inclusion complex. This RMSD clustering is a technique to group the same orientations based on the distance metric of RMSD coordinates of ED and βCDs using the DBSCAN density-based clustering algorithms with minimum number of points set to 25 [40]. Furthermore, the water accessibility of each inclusion complex was studied by number of contacts, solvent accessible surface area (SASA) and radial distribution function (RDF) using focused oxygen atoms in ED. Moreover, we studied the radius of gyration (R_{gyr}) of all systems and conformational changes of host βCDs upon inclusion complexation in relation with PES calculations. Finally, to explore the binding affinities between host and guest molecules, the molecular mechanics/generalized Born surface area (MM/GBSA) calculations by py.module [41] were applied using the 10,000 snapshots extracted from the last 100-ns simulations.

3. Results and discussion

3.1 Binding orientations of ED inside the cavity of βCDs

After 100-independent docking runs, it was found that ED showed two favorable orientations inside hydrophobic βCDs interiors. The resorcinol ring of ED inserted into βCDs cavities was stated as R-form, while *m*-cresol ring insertion was named as C-form (**Fig. 1C**). It should be noted that the attachment of ED outside the

hydrophobic cavity of βCDs (accounted for ~1-4%) was excluded in this work because we focused on the inclusion complexation of ED with βCDs. The %DCs of R-form or C-form summarized in **Fig. 1D** highlights that the population of C-form insertion was higher in ED/SBEβCD and ED/HPβCD inclusion complexes, related with the NMR data of Neng Qiu's group for ED toward HPβCD's cavity [13]. On the other hand, R-form orientation was dominantly inserted in the native βCD. The interaction energies between R-form (-23 to -28 kcal/mol) and C-form (-22 to -27 kcal/mol) are somewhat similar; thus, both orientations were selected to be the initial structures for MD simulations.



	% Docked conformations (%DCs)	
	R-form	C-form
ED/βCD	65	31
ED/HPβCD	41	58
ED/SBEβCD	44	54

Fig. 1. (A) The chemical structure of ED consisting of resorcinol ring and *m*-cresol ring. (B) The representative structure of native βCD and its substitutions. (C) The molecular docked orientation of ED complexed with βCD. (D) The corresponding %DCs of all studied ED/βCDs inclusion complexes.

3.2 System stability

To evaluate the system stability, RMSD was determined, and the obtained data are plotted in **Fig. 2**. Throughout the simulations, ED/HPβCD and ED/SBEβCD inclusion complexes showed the similar trends of system stability with RMSD fluctuations at 2-4 Å and 4-6 Å, respectively. Unexpectedly, the dissociation of ED occurred in ED/βCD complexes, especially C-form in MD #1 and MD #3, before achieving its equilibrium after 300ns with respective RMSD value at 2-5Å. The immediate formation of inclusion complexes in ED/modified βCDs was likely due to the influence of substituted groups which could stabilize the guest molecule than the parent βCD [42-44], and the enlarged openings in modified-βCDs compared to native βCD provide the guest molecule an easy entry [43]. Each studied system shows the system stability after 300ns; thus, further analysis was performed on the last 100-ns MD trajectories (400-500ns).

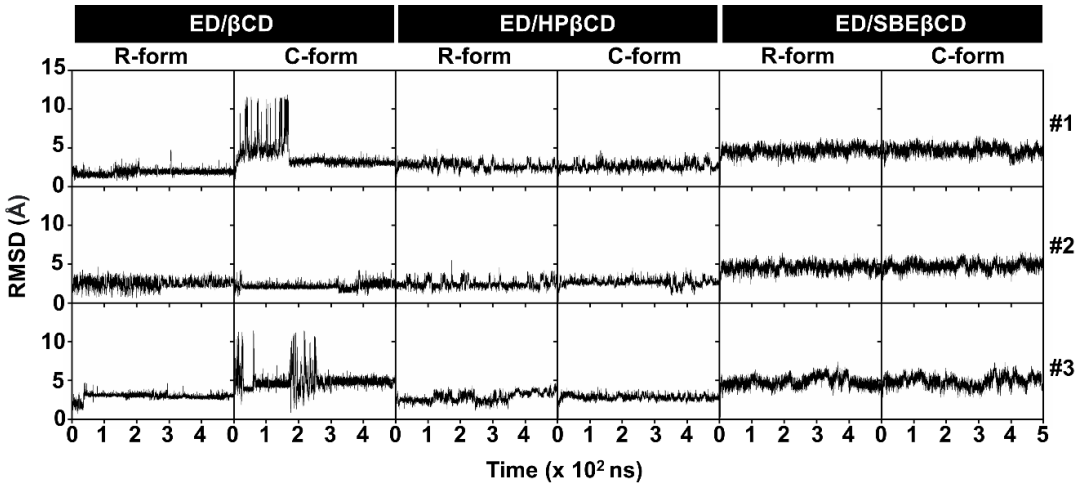


Fig. 2. All-atom RMSD plots of ED/βCDs inclusion complexes in both orientations (R-form and C-form), depicted over 500 ns in three replicates.

3.3 ED mobility inside hydrophobic cavity

To observe the mobility of ED inside hydrophobic cavity, the study of distance analysis was considered by measuring the distance between center of mass (C_m) of

primary rim of βCDs toward C_m of rings of ED, d[$C_{m(primary\ rim)}$ - $C_{m(ring)}$] as depicted in **Fig. 3**, whereas the distance measurement from C_m of secondary rim of βCDs to $C_{m(ring)}$ of ED, d[$C_{m(secondary\ rim)}$ - $C_{m(ring)}$] is illustrated in **Fig. S3**, without taking into account the functional substituents. Note that the dimension of unmodified βCD is around 7.9 Å [45].

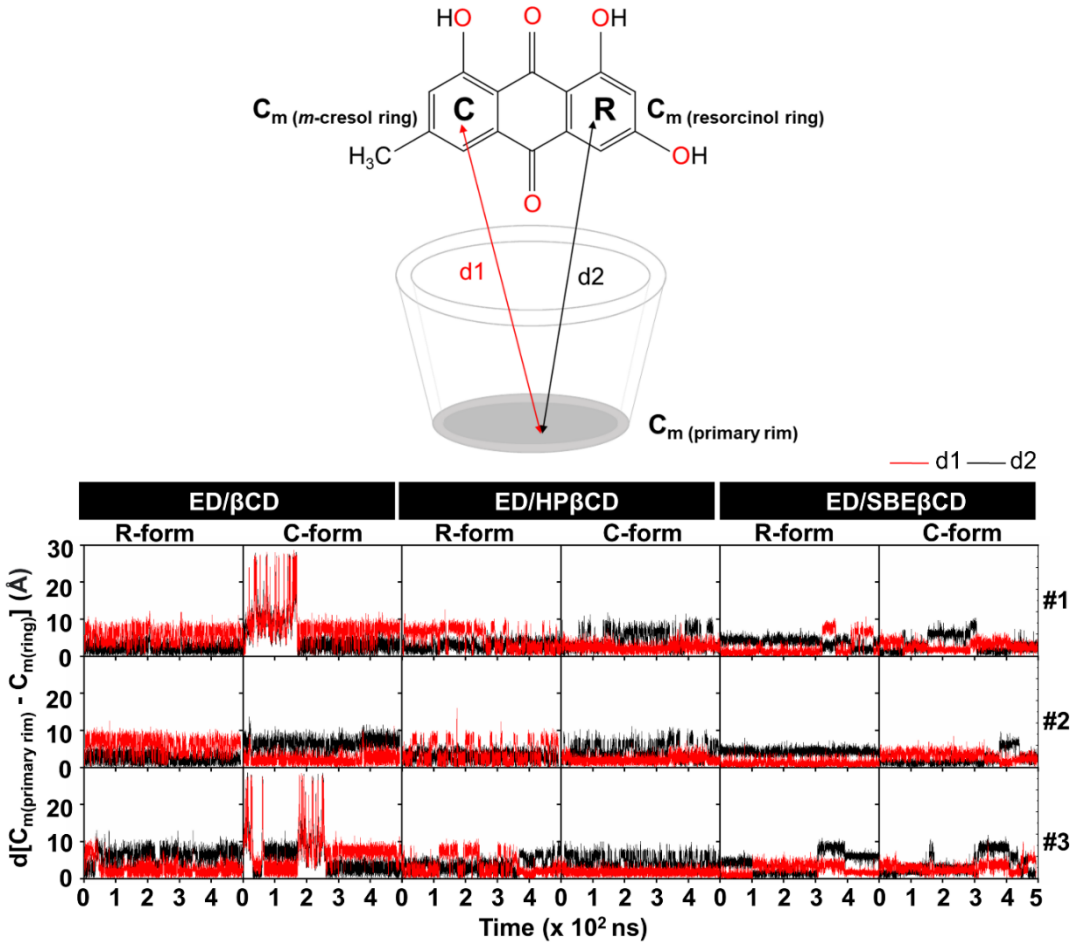


Fig. 3. The representative structure of distance measurement from $C_{m(primary\ rim)}$ of βCDs to $C_{m(rings)}$ of ED, d[$C_{m(primary\ rim)}$ - $C_{m(ring)}$] and the distance analysis plotted over 500 ns, in which d[$C_{m(primary\ rim)}$ - $C_{m(C\text{-}ring)}$] (d1) and d[$C_{m(primary\ rim)}$ - $C_{m(R\text{-}ring)}$] (d2), are shown in red and black, respectively.

As shown in **Fig. 3**, in MD#3 of ED/βCD (R-form), the initial R-form orientation was converted to C-form at ~50 ns and retained this orientation until the end of simulation, whereas the MD#1 and MD#2 were found to be stable in R-form along the entire simulation. Similarly, in MD#1 and MD#3 of ED/βCD (C-form), ED moved out of the βCD cavity at the first 250 ns and then adopt to the R-form orientation after 300 ns, while the MD#2 was found to be stable in C-form along the entire simulation. Therefore, from six MD runs of ED/βCD, R-form and C-Form insertions were counted as four and two, respectively. In some studied systems, especially in MD#3 of ED/SBEβCD, the flipping of ED inside the host cavity is difficult to determine because d1 and d2 exhibited the same distance at around 5 Å. To ensure the structural changes of the formed inclusion complexes, we further analyzed the distance measurement from the center of secondary rim of βCDs to the respective ring of ED and the data is plotted in **Fig. S3**. Thus, both distance results from **Fig. 3** and **Fig. S3** can give us the conformational changes along the simulations whether C-form or R-form is stable for each system. Based on the MD trajectories of the last 100-ns of three MD runs of ED/HPβCD and ED/SBEβCD in each orientation, two- and four-times insertions of R-form, and C-form were observed, thus we analyzed the trajectories based on the structural information of the last 100-ns.

To support this finding, RMSD clustering over 10,000 snapshots taken from last 100-ns was calculated and plotted in **Fig. 4**. There are three groups of inclusion complexes in which cluster 1 was the major population for all studied systems. The second most populated cluster is defined as cluster 2 and the least populated complex as cluster 3, respectively. We found that the percent population among clusters was noticeably different. However, there are no conformational changes between R-form and C-form in all clusters. Similar to the distance analysis, R-form insertion was populated four times in ED/βCD inclusion complexes, whilst C-form orientation was the major cluster in ED/HPβCD and ED/SBEβCD inclusion complexes. Moreover, it

was observed that ED positioned firmly inside βCDs core in all studied systems, supporting the inclusion complex formation of ED toward βCDs. Therefore, based on the distance analysis and RMSD clustering, we finally grouped R-form and C-form of respective system and analyzed the data according to the pre-grouped clusters.

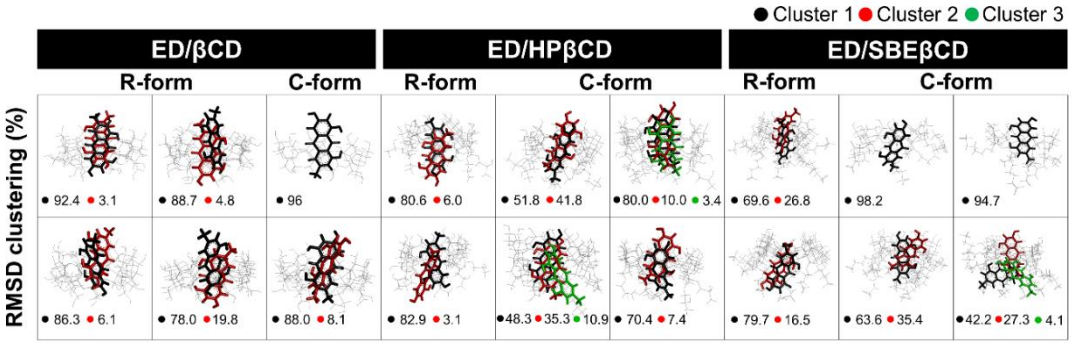


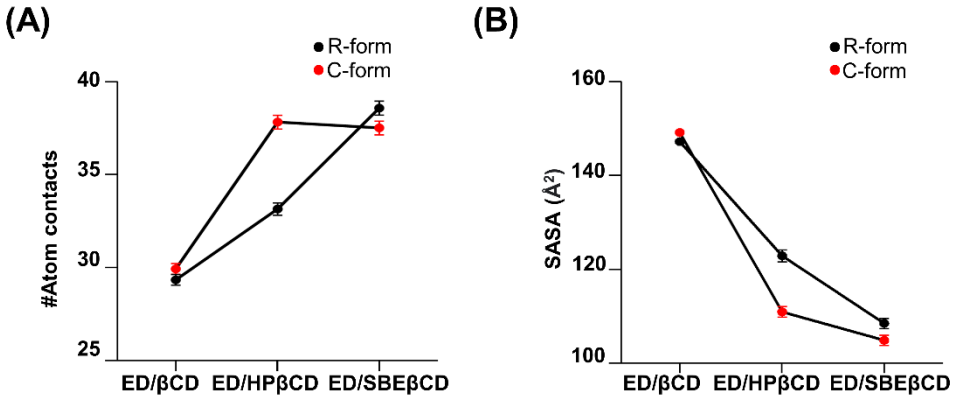
Fig. 4. Representative structures of host-guest inclusion complexes clustered by distance analysis. The population of each cluster was described in the percentage value.

3.4 Atomic contacts and water accessibility toward inclusion complex

The atom-atom contacts involved in the ED/βCD(s) inclusion complexations was calculated using the probe radius distance within 3 Å cutoff, i.e., any contacting atoms closer than 3 Å between atoms in ED and atoms in βCD(s) were counted. The high number of contacts refers to the favorable and dense interaction, and the low value represents the low interaction between host and guest molecules [46]. The obtained results, analyzed over last 100-ns (**Fig. 5A**), revealed that the ED/SBEβCD inclusion complex (R-form) displays the highest contacts with 38.58 ± 0.38, followed by C-form orientation with 37.51 ± 0.37. The second highest contacts were observed in C-form and R-form of ED/HPβCD complex with 37.82 ± 0.37 and 33.15 ± 0.33, respectively. On the contrary, the lowest number of contacts was witnessed in ED/βCD complex with 29.92 ± 0.29 (C-form) and 29.34 ± 0.29 (R-form). Altogether, the number of contacts was ranked in the order of ED/SBEβCD > ED/HPβCD > ED/βCD

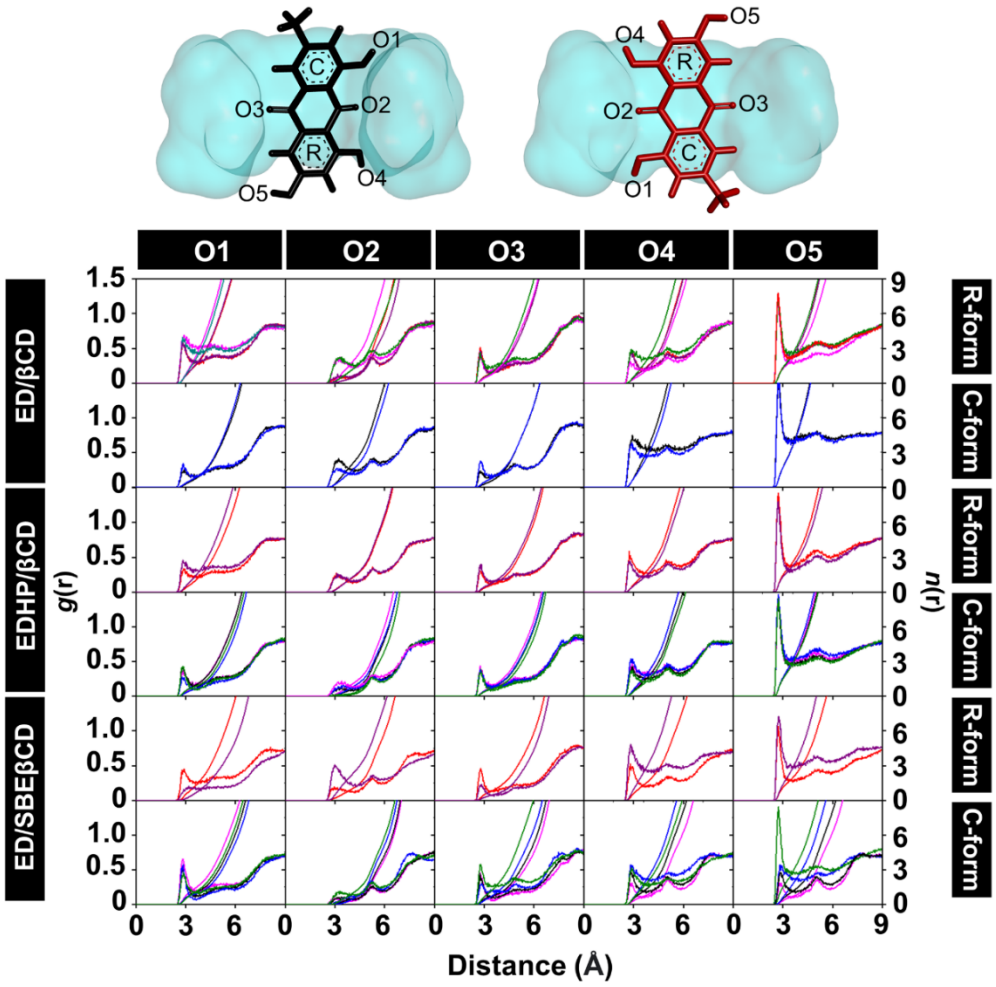
inclusion complexes, meaning that ED is likely to interact with SBEβCD better than the other focused hosts; thus, SBEβCD could serve as the most suitable host for ED.

To support this finding, we further analyzed the solvent accessibility of all studied systems using ED as atomic radii for solvent-exposed area (**Fig. 5B**) based on assumption that when ED forms inclusion complex with βCD(s), the water accessibility toward ED molecule should be reduced. As expected, the lowest SASA value of 104.85 ± 1.04 $Å^2$ was observed in ED/SBEβCD inclusion complexes especially in C-form orientation. The moderate occupation of surrounding water molecules was observed in ED/HPβCD inclusion complex with 122.87 ± 1.22 $Å^2$ under C-form orientation and the highest average SASA was observed in ED/βCD complexes with 149.18 ± 0.12 $Å^2$ (C-form), indicating that dispersing water molecules are more likely to approach to native βCD inclusion complexes which may be due to the lack of substitutions to protect guest molecule compared to the modified-βCDs. Altogether, the SASAs were ranked in the order of ED/βCD > ED/HPβCD > ED/SBEβCD, in line with the contact calculations [47], supporting the fact that SBEβCD could serve as the most appropriate host for ED.



	Number of contacts		SASA	
	R-form	C-form	R-form	C-form
ED/βCD	29.34 ± 0.29	29.92 ± 0.29	147.21 ± 0.44	149.18 ± 0.12
ED/HPβCD	33.15 ± 0.33	37.82 ± 0.37	122.87 ± 1.22	110.94 ± 1.11
ED/SBEβCD	38.58 ± 0.38	37.51 ± 0.37	108.45 ± 1.08	104.85 ± 1.04

Fig. 5. (A) The number of contacts and (B) SASA of all studied inclusion complexes calculated over last 100-ns simulations. Data are expressed in mean ± standard error of mean (SEM), n=3.



n(r)	ED/βCD		ED/HPβCD		ED/SBEβCD	
	R-form	C-form	R-form	C-form	R-form	C-form
O1	2.43 ± 1.21	0.85 ± 0.56	1.37 ± 0.97	0.95 ± 0.47	0.74 ± 0.52	1.29 ± 0.64
O2	1.11 ± 0.55	2.15 ± 1.52	1.69 ± 1.19	0.28 ± 0.01	1.30 ± 0.91	0.26 ± 0.03
O3	0.73 ± 0.36	0.64 ± 0.45	0.78 ± 0.55	0.84 ± 0.42	0.78 ± 0.55	1.03 ± 0.51
O4	1.35 ± 0.67	2.75 ± 2.65	1.74 ± 1.23	1.64 ± 0.82	3.03 ± 2.14	2.04 ± 1.02
O5	2.30 ± 1.15	3.10 ± 2.19	2.44 ± 1.72	3.03 ± 1.51	2.73 ± 1.93	3.60 ± 1.80

Fig. 6. (Top) RDF of water around the O1-O5 atoms of ED encapsulated in βCDs cavities over last 100-ns and (Bottom) the average integration number, *n(r)*, up to the first minimum derived from RDF plots, corresponding to the number of water molecules pointing toward the focused oxygen atoms of ED.

Next, the radial distribution function (RDF, *g(r)*) was used to visualize the surrounding water molecules (integration number, *n(r)*) within a spherical radius *r* of the ED's oxygen atoms (O1-5). The average number of water molecules, *n(r)*, is summarized in **Fig. 6**.

Noticeably, the low hydration peaks were observed in O2 and O3. This might be due to the position of these oxygen atoms which are located inside the hydrophobic core of βCDs [13, 48, 49]. Especially in the case of O2 oxygen atom in C-form of ED/HPβCD and ED/SBEβCD, the very weak hydration peak (*n(r)*=0.26-0.28) was observed, indicating a rather low water accessibility toward C-ring of ED. In addition, O4 and O5 oxygen atoms of ED (*n(r)*=1.64-3.60) are more exposed to surrounding water molecules than O1 (*n(r)*=0.95-1.37) by providing the distinctive peaks for almost all systems. These results indicate that C-ring of ED point to the hydrophobic cavity, while R-ring positions near the primary or secondary rims of modified-βCDs. The reason could be due to the hydrophobic moiety of C-ring under the influence of CH_3 side group compared to focused oxygen atom at R-ring. However, in ED/βCD inclusion complexes, especially R-form, O1, O4, O5 are equally targeted by water molecules. It seems that the presence of side chain in modified βCDs provides a large molecular shield to the guest molecule from the attack of surrounding water molecules compared to the parent structure. RDF analysis provides us the information of how water approach to the focused oxygen atoms of ED and the obtained results were in good correlation with number of contacts and SASA analysis. Taking all findings discussed above into consideration, it suggests that SBEβCD could be the most suitable host for ED, since ED/SBEβCD complex possesses the highest atomic contact

and binding affinity as well as the lowest SASA compared to the other studied systems.

3.5 System compactness

The results from number of contacts and SASA suggested that ED is able to form inclusion complex with βCDs. To support this finding, we further examined the system compactness using the calculation of radius of gyration (R_{gyr}) which represents the mass-weighted scalar length of each atom from center of mass of the molecule [50]. The resulting R_{gyr} values from the last 100-ns simulations are depicted in **Fig. 7**. The R_{gyr} values of unbound βCD, HPβCD, and SBEβCD were 6.25 ± 0.19, 6.85 ± 0.43, and 8.03 ± 0.31 Å, respectively, and these values of R_{gyr} for βCD and HPβCD are in similar trends with previously reported by Yong *et al.* [51] using MD simulations and by K. Kerdpol *et al.* [21] using replica exchange molecular dynamics (REMD) simulations. The R_{gyr} values of ED/βCD, ED/HPβCD, and ED/SBEβCD inclusion complexes are 6.05 ± 0.05, 6.41 ± 0.06, and 7.48 ± 0.07 Å, respectively. Obviously, the R_{gyr} values in studied complexes are decreased because formation of inclusion complex induces the decreased conformation flexibility of βCDs as a result of interaction with ED. There are no significant differences in R_{gyr} values between R-form and C-form of each system. Overall, the molecular compactness study further supports the inclusion complexation of ED toward βCDs.

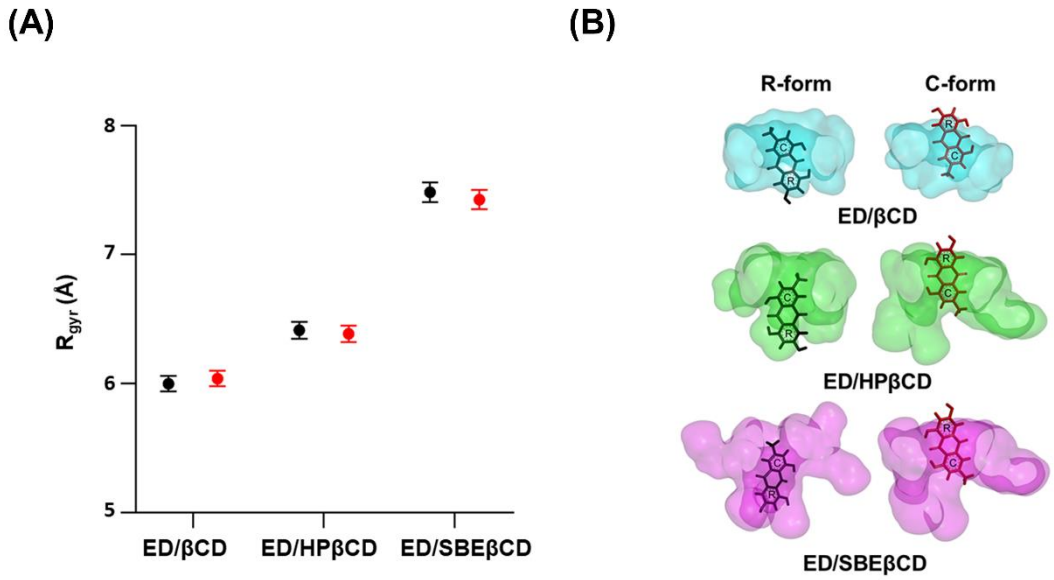


Fig. 7. (A) R_{gyr} plot of all studied inclusion complexes calculated over last 100-ns. Data are shown as mean ± SEM. (B) The surface representation of all studied models: ED/βCD in cyan, ED/HPβCD in green, and ED/SBEβCD in pink.

3.6 βCDs conformations in relation with PES calculations

The conformational changes of host upon ED complexation were further investigated by calculating (a) the distance of hydroxyl groups on secondary rim of βCD ($d_n[O3_{(n)} - O2_{(n+1)}]$), whose values was considered <3.5 Å corresponding to the possibility of intramolecular hydrogen bond formation between adjacent oxygen atoms at wider rim and (b) the distance of glycosidic oxygen atoms ($d_n[O4_{(n)}-O4_{(n+1)}]$). The resulting data was converted to the PES using the following Poisson-Boltzmann equation, where k_B is Boltzmann constant, T is the temperature (298K), and P(x,y) is the probability of $d_n[O3_{(n)} - O2_{(n+1)}]$ (x) and $d_n[O4_{(n)} - O4_{(n+1)}]$ (y) and shown in **Fig. 8**.

$$F(x,y) = -k_B T \log[P(x,y)]$$

The three local minima areas in unbound state were illustrated in **Fig. S2** in the supplementary data. The PES studies of free forms reveal that there are three local minima areas (M1, 2-3 Å, M2, and M3, 5.5-6.5 Å) for parent βCD and HPβCD while only two local minima (M1 and M2) were observed in SBEβCD, in which M1 area is

likely to be the stable region within the range of hydrogen bond formation and M2 and M3 regions represent the distortion of glucose units. Upon encapsulation process, the stable conformation of βCDs was achieved by showing the distinctive population of M1 region in all studied complex as a result of hydrogen bond formation between $O3_{(n)}$ and $O2_{(n+1)}$, within approximately 2-3 Å. In addition, M3 region with lengthened distances at both axes which was detected in βCD and HPβCD is completely disappeared in inclusion complex state. The presence of M2 (approximately 5.5-6.5 Å) in ED/βCD and ED/HPβCD but not in ED/SBEβCD indicates SBEβCD as the best host for ED. These H-bond-generated structural changes of βCDs upon inclusion complexation are in good agreement with previous study [16]. To conclude, βCDs conformations in relation with PES calculations upon inclusion complexation further support ED/SBEβCD as the most stable inclusion complex, with is consistent with MM/GBSA based calculations as discussed later.

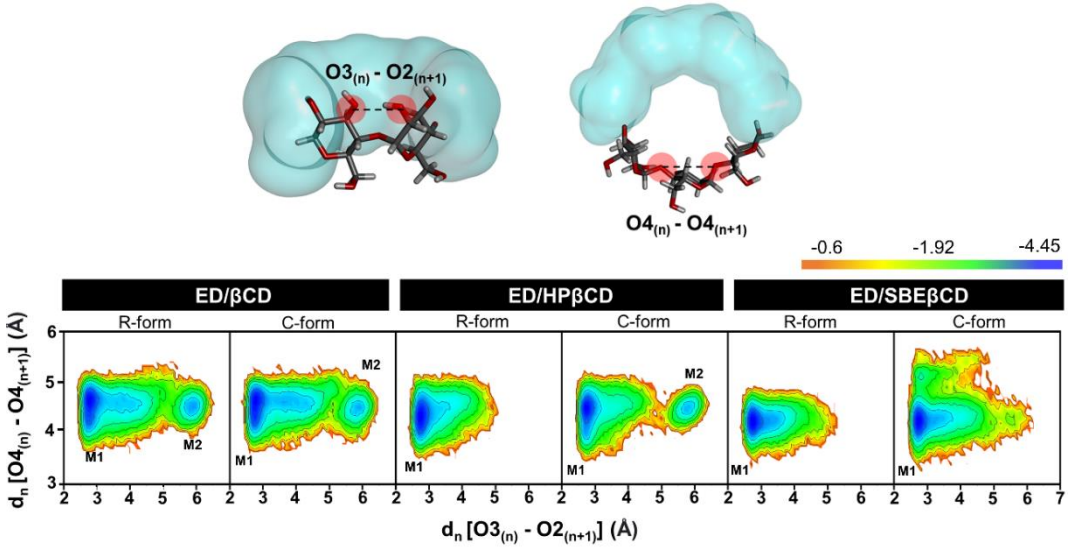


Fig. 8. PES of all studied βCDs in complex with ED calculated over last 100-ns.

3.7 Binding free energy of studied inclusion complexes

To investigate the binding affinity between host and guest molecules in all studied inclusion complexes, MM/GBSA based binding free energy (ΔG_{bind}) calculations were applied on 10,000 snapshots extracted from last 100-ns of MD

simulations. The van der Waals interaction (ΔE_{vdW}), electrostatic attraction (ΔE_{ele}) and molecular mechanics energy (ΔE_{MM}) (the summation term of ΔE_{vdw} and ΔE_{ele}) was summarized in **Fig. 9A**. As expected, ΔE_{vdW} was the major force contributed to form inclusion complexes and the resulting values were approximately -27 kcal/mol for ED/βCD, -29 to -32 kcal/mol for ED/HPβCD, and -32 to -34 kcal/mol for ED/SBEβCD, respectively in good agreement with other reports showing that ΔE_{vdW} is the main driving force for the formation of inclusion complexes [20, 52-54]. The calculated ΔG_{bind} values were depicted in **Fig. 9B** which can be ranked in the order of ED/SBEβCD > ED/HPβCD > ED/βCD, with approximately -5.05 to -6.18 kcal/mol, -3.2 to -3.8 kcal/mol, and 0.11 to 0.28 kcal/mol, respectively. The calculation results from this study show similar trends with previous reports demonstrating that docetaxel and chrysin in complex with SBEβCD showed the highest stability, followed by those in complex with HPβCD and βCD, respectively [55, 56]. Supportively, several studies have proved that SBEβCD bears higher inclusion ability due to the presence of long hydrocarbon chain in structure which extends the hydrophobicity and enlarges the volume of parent cavity [26, 57]. Taking account into all *in silico* data, it was convinced that βCD derivatives, in particular SBEβCD, could serve as the most suitable host for ED through molecular encapsulation.

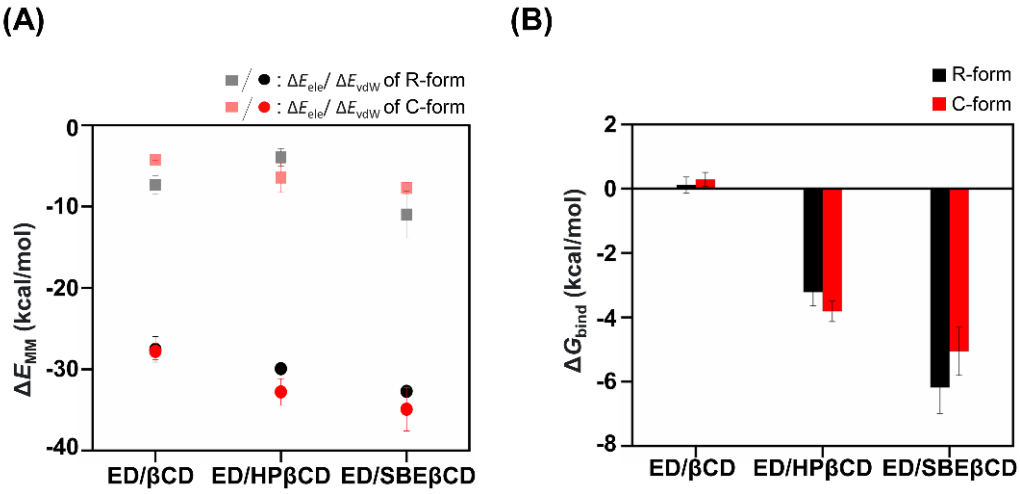


Fig. 9. (A) The molecular mechanics energy components and (B) ΔG_{bind} of all studied systems calculated with MM/GBSA method.

4. Conclusions

In this work, we presented 500-ns MD simulations of ED in complex with βCDs. Overall, ED is able to perform inclusion complex with native βCD after 300 ns, while ED in complex with the modified βCDs achieved the system stability quickly just after heating up step. Inclusion complex formation between ED and βCDs occurred in two possible orientations: R-form and C-form. The distance analysis together with RMSD clustering revealed that R-form insertion was dominated in ED/βCD complex, whereas C-form orientation was the major conformation for the remaining studied complexes. Based on water accessibility and atomic contact analyses, βCD derivatives, especially SBEβCD, show better encapsulation efficiency with ED than the parent βCD. The PES calculation revealed that the noticeable population of stable M1 region in all systems, whereas the distortion of glucose units (M2) was observed in ED/βCD and ED/HPβCD systems. Supportively, MM/GBSA calculation showed that the lowest ΔG_{bind} values were observed in ED/SBEβCD followed by ED/HPβCD and ED/βCD, respectively. In conclusion, all structural findings suggest that βCD derivatives, especially SBEβCD, could serve as the most promising drug carrier for ED to improve its water solubility and stability.

CRediT authorship contribution statement

Amy Oo: Writing - original draft, **Khanittha Kerdpol:** Writing - review & editing, **Panupong Mahalapbutr:** Conceptualization, Writing - review & editing, Supervision, **Thanyada Rungrotmongkol:** Conceptualization, Writing - review & editing, Supervision.

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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CHAPTER II

MANUSCRIPT II

In vitro studies on inclusion complexation of emodin with various β-cyclodextrin derivatives

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Abstract

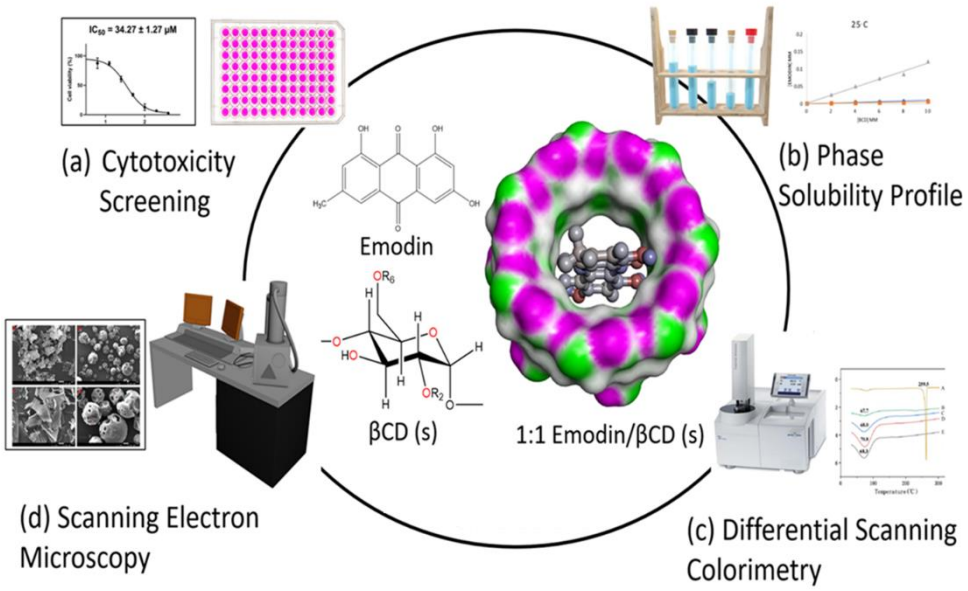
An enormous amount of research, ranging from co-solvency to nano-crystallization, has been carried out to enhance the water solubility of poorly soluble drugs. Out of ample techniques, complexation with β-cyclodextrin (βCD) has gained a lot of research attention due to its unique structure where many kinds of lipophilic guest molecules are entrapped into its hydrophobic cavity. Emodin (ED, 6-methyl-1,3,8-trihydroxyanthraquinone) stands out for its profound biological properties. The anti-cancer activities of ED toward various cancer cell lines via DNA intercalation, cell cycle arrest and induction of apoptosis have been well reported. However, its usage in pharmaceutical applications has been restricted due to its poor aqueous solubility. To address this issue, we performed a series of complexation of ED with βCD and its derivatives: hydroxypropyl-β-cyclodextrin (HPβCD), 2,6-di-O-methyl-β-cyclodextrin (DMβCD), and sulfobutylether-β-cyclodextrin (SBEβCD), to identify the most promising drug carrier for emodin with regards to water solubility enhancement and augmented biological properties through host-guest complexation. The AL-type diagram indicates the 1:1 stoichiometry between ED and βCDs. ED/DMβCD show the highest stability with K_C values of 1500 M^{-1} at 25ºC and water solubility with 113.86ug/mL at 30ºC. The micrographs by scanning electron microscopy characterize the inclusion complex formation between host and guest. The potent cytotoxicity of ED and all studied complexes were investigated using cholangiocarcinoma cell lines and it was observed that ED/DMβCD complex shows the most promising inhibition with the IC_{50} values of 52.78 ± 1.57 and 47.00± 0.58 µM toward KKU-213A and KKU-213B, respectively.

Keywords: Emodin, β-cyclodextrin, hydroxypropyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin, di-O-methyl- β-cyclodextrin, inclusion complex, cholangiocarcinoma cells.

Highlight

- The aqueous solubility of ED can be enhanced by inclusion complexation with β-cyclodextrin and its derivatives.
- The best stability, the highest water solubility, and the most promising inhibition pattern was observed in ED/DMβCD complex.

Graphical abstract



1. Introduction

In drug discovery and development, solubility of active compounds is considered to be an essential factor to deliver the desired amount into systemic blood circulation for pharmacological response to be shown [58]. The poor water solubility of potential drug candidates has often left to failure [59] and the previous study by Benet *et al.* [60] reported that up to 90% of the potent compounds in the drug development suffer from the low water solubility. The optimized bio pharmaceutically relevant solubility of drug substances are in greater need to substantially develop them into pharmaceutical market. Various approaches have been utilized to resolve the poor water solubility of lipophilic molecules such as particle size reduction [61], solvency [62], hydrotropy [63], salt formation [64], solid dispersion [65], and inclusion complexation with cyclodextrin (CD) [66]. Several studies reported that inclusion complexation with CD can notably increase the aqueous solubility, the dissolution rate, and the stability of many insoluble compounds [67-70]. CD is a naturally occurring cyclic oligosaccharide from starch degradation by glucotransferase enzyme [71, 72], possessing the unique truncated cone shape structure with relative hydrophobic inner cavity and hydrophilic outer surface. Depending on the numbers of glucose units on the cyclic skeleton, there are three main types of CD; alpha-cyclodextrin (αCD), beta-cyclodextrin (βCD) and gamma-cyclodextrin (γCD) with 6, 7, and 8 glucose units respectively. In recent years, the molecular encapsulation with βCD becomes an interesting option owning to its desirable drug loading capacity, commercial availability and biocompatibility [15, 73]. βCD presents the molecular encapsulation property with the guest molecule mainly through Van der Waals interaction [16]. In spite of having favorable features,

the nephrotoxicity and the low aqueous solubility of βCD leads to the structural modification of parent structure. Radom substitutions of hydroxy groups at the primary and secondary rims result in dramatic improvements of the water solubility and lower renal toxicity [19-21]. Among pharmacologically interested βCDs, hydrophilic derivatives such as hydroxypropyl-β-cyclodextrin (HPβCD), di-O-methyl-β-cyclodextrin (DMβCD), and sulfobutylether-β-cyclodextrin (SBEβCD) (**Fig. 1A**) become the commonly used derivatives [74].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), ED, belongs to the naturally occurring anthraquinone family which is made up of more than 170 natural compounds [75]. ED has been identified in the diverse plant families such as Bignoniaceae and Simaroubaceae [76] in tropical regions, and Polygonaceae [77] and Saxifragaceae [78] in temperate regions. ED is mainly distributed in plant vegetative organs such as stem [75], and reproductive organs such as flower, fruit, seeds, pods [79]. ED has a tricyclic aromatic skeleton with hydroxyl and methyl group at C3 and C6 positions respectively (**Fig. 1B**). ED has been used as a laxative compound since ancient times and it was reported that the presence of hydroxyl groups in C1 and C8 of the aromatic ring system plays the important role for the purgative action of ED [80]. Among various natural anthraquinones, ED stands out for its diverse biological functions including anti-cancer, anti-bacterial, anti-viral, anti-inflammatory, anti-ulcerogenic, immunosuppressive and chemopreventive effects [81-84]. The anti-inhibitory effect of ED in various types of cancer cells such as lung cancer [85, 86], breast cancer[87, 88], cervical cancer [89], colon cancer [90, 91], gallbladder cancer [92], pancreatic cancer [93], hepatic carcinoma cells [94], and leukemia [95], have been reported. The molecular inhibition mechanisms of ED through induction of

apoptosis has been well documented in breast cancer through mitochondrial signaling pathway [87], in cervical cancer via intrinsic mitochondrial and extrinsic death receptor pathway [89], in pancreatic cancer through declining the mitochondrial membrane potential [93] and in lung cancer through ROS-dependent mitochondrial signaling pathway [96]. ED was previously reported to inhibit the activity of several protein kinases such as cAMP-dependent protein kinase (PKA), protein kinase C (PKC), casein kinase I (CKI) and II (CKII) [97], and protein kinase B (AKT), mitogen-activated protein kinase (MAPK) [98], HER-2 tyrosine kinase [99], integrin-linked kinase (ILK) [100] and phosphatidylinositol 3-kinase [101]. Moreover, the ability of ED to intercalate DNA, and to selectively interact with minor grove of DNA hinder the cell cycle arrest. [7, 102]. However, belonging to the biopharmaceutical classification system (BCS) type II [103], ED is being considered as an unsatisfactory chemotherapeutic agent due to its poor aqueous solubility with the relatively poor bioavailability. It was previously reported that the water solubility of ED can be enhanced by inclusion complexation with βCD [104] and HPβCD [13].

In the present study, *in vitro* studies on inclusion complexation of ED with βCD and its hydrophilic derivatives; HPβCD, DMβCD, and SBEβCD were performed to find the best host molecule for ED in order to improve its aqueous solubility, stability and the anti-cancer potential towards cholangiocarcinoma cell lines (KKU-213A and KKU-213B).

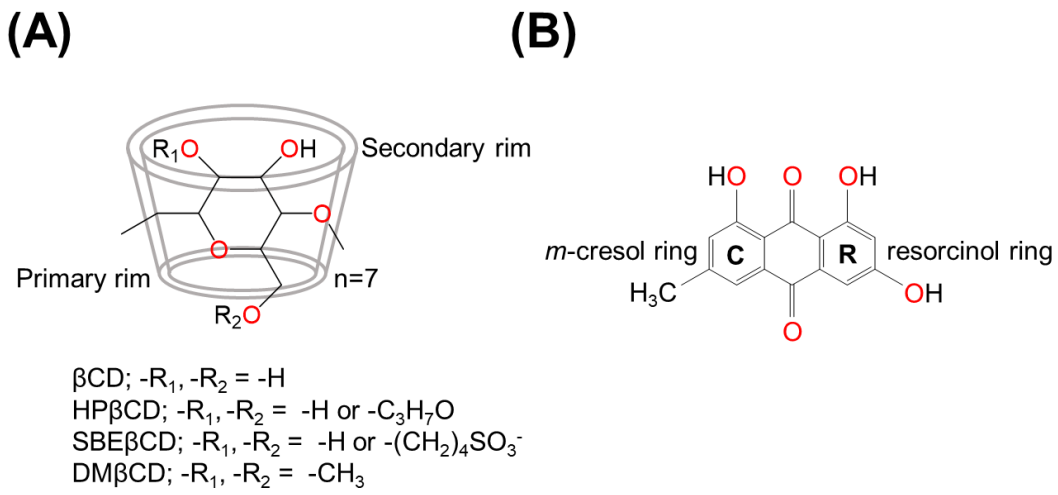


Fig. 1. Chemical structures of (A) βCD and its substitutions and (B) ED

2. Materials and methods

2.1. Materials

ED was obtained from Professor Dr. Chutima Kuhakarn's laboratory from Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. HPβCD, βCD were purchased from TCI (Nihonbashi-honcho, CK, Tokyo) whereas SBEβCD and DMβCD were purchased from Sigma-Aldrich (St. Louis, MO, USA). Note that the degree of substitution (DS) for HPβCD is 0.5-1.3 HP groups per glucose unit and that for SBEβCD is 2, with that of DMβCD being 14.

2.2. Phase solubility study

To study the thermodynamics behaviors of ED, phase solubility testing was performed using Higushi and Connors [105] under four different temperatures; 25 ◦C, 30 ◦C, 37 ◦C, and 45 ◦C. Briefly, excess amount of emodin (3mM) dissolved in ethanol was added to logarithmic concentrations of aqueous βCDs solutions (0-10

mM). The mixtures were shaken at 250rpm at each temperature for 72 h. The suspension was centrifuged at 12,000 rpm for 15min. The resulting supernatant was then filtrated and the amount of represented ED was measured at 438nm. The stability constant (K_C) was then calculated using equation $K_C = \frac{Slope}{[S0(1-slope)]}$, where *S0* is y-intercept. To explore the thermodynamics behaviors of ED, Van't Hoff plot based on equation $lnK_C = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$ [106], including enthalpy (ΔH) and entropy (ΔS), was used. The experimental Gibbs free energy ($\Delta G_{bind,exp}$) was calculated using equation $\Delta G_{bind,exp} = \Delta H - T\Delta S$. This experiment was repeated three times and the results were reported in mean ± standard error means (SEM) values.

2.3. Inclusion complex preparation

Freeze-drying method was used to prepare inclusion complexes of ED/βCDs. 1:1 stoichiometric ratio of ED and studied βCDs was recorded from the phase solubility testing. Briefly, equal molar ratio (0.5mM) of ED and βCDs were prepared in 50mL distilled water. The mixtures were magnetically shaken at room temperature for 24 h, followed by centrifugation of the suspension at 12,000 rpm for 15min. The resulting supernatant was filtered through a 0.45μm membrane filter. The obtained solution was then frozen at -80 ◦C overnight. The frozen samples were subsequently lyophilized using Labconco FreeZone (4.5L) for 3 days. Physical mixtures were also prepared using the same molar ratio. The resulting freeze-dried powders and physical mixtures were then stored in desiccator for future use.

2.4. Inclusion complex characterization

2.4.1 Scanning electron microscope (SEM)

Using Scanning Electron Microscope (Carl Zeiss Leo 1450 Vp), the surface morphology of pure ED, pure βCDs, the freeze-dried inclusion complexes and the physical mixtures, was explored. Briefly, ~1-2mg of the studied samples were firstly gold-coated and examined under the 1 K.X magnification. The images were captured at an accelerating voltage of 20kV.

2.4.2. Differential scanning calorimetry (DSC)

Using DSC analyzer (NETZSCH DSC 204F1 Phoenix), the thermal behaviors of pure ED, βCD(s) and the inclusion complexes were studied. Briefly, ~1–2 mg of prepared freeze-dried powder of each mixture was placed in sealed aluminum pans and scanned over a range of 25 ◦C to 300 ◦C at a rate of 10 ◦C /min with a coolant nitrogen flowrate of 25mL/min and DSC thermograms were recorded.

2.5. Determination of solubility

Excess amount of pure ED and its inclusion complexes were added to 500ul of distilled water. The mixtures were then stirred at 30◦C for 24 h. The resulting suspension was subsequently filtered through a 0.45 μm filter and solubility of ED was checked using Biotek Synergy H1 hybrid multi-mode reader at 438 nm.

2.6. Determination of drug content

Accurately weighted 2mg of ED and its inclusion complexes were dissolved in 100µl of ethanol and the absorbance was measured at 438nm using Biotek Synergy H1 hybrid multi-mode reader.

2.7. Cell culture and cytotoxicity

Cell culture and cytotoxicity screening (MTT) assay were performed at Associate Professor Dr. Atit Silsirivanit's laboratory at Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand. The cholangiocarcinoma cell lines (KKU-213A and KKU-213B) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin, 100µg/mL streptomycin, and 10% fetal bovine serum and subsequently kept at 37°C in a humidified 5% CO_2 incubator. Cultured cells were then seeded into 96-well plates at a density of 1000 cells/well and allowed to adhere by overnight incubation. Cells were then treated with 100µl of serial concentrations of ED and the prepared inclusion complexes (0.3, 1, 3, 10, 30, and 100 µM) for 48h. Ten µl of prepared MTT solution (5mg/mL) was then added to each well and incubated at 37°C in a CO_2 incubator for 3 h. The culture medium was thereafter withdrawn and 100µl of DMSO solution was then added to dissolve the formazan salt. The absorbance was recorded at 540nm using Tecan Sunrise absorbance microplate reader. The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 7 software. Experiment was performed in triplicates and the results were described with mean ± SEM.

2.8. Statistical analysis

The quantitative data are expressed as mean ± standard error of mean (SEM) of three independent experiments. The differences between groups were determined using t-test (and nonparametric tests) built in GraphPad Prism 7 software. The p value of 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Phase solubility profile and thermodynamic behaviors

The phase solubility diagrams of ED/βCDs complexes at four different temperatures; 25, 30, 37 and 45 ºC are plotted in **Fig. 2**. The obtained results show the solubility of ED can be enhanced by inclusion complexation with βCDs in a concentration-dependent manner indicating the1:1 stoichiometry between host and guest (AL-type). The solubility of studied complexes at each temperature was ranked in the order of ED/DMβCD > ED/HPβCD > ED/SBEβCD > ED/βCD.

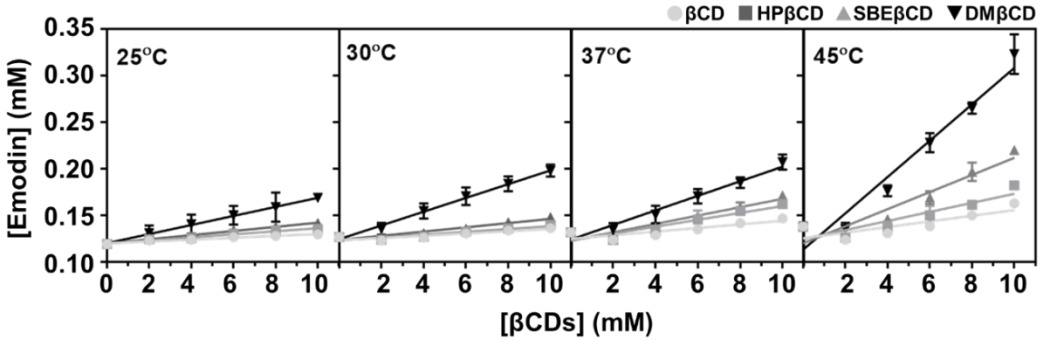


Fig. 2. The phase solubility profiles of ED in complex with βCDs at different temperatures; 25, 30, 37, and 45 ºC.

Table. 1. The stability constants of all studied complexes at different temperatures.

Temperature (°C)	Stability constant (K_C in M^{-1})			
	ED/βCD	ED/SBEβCD	ED/HPβCD	ED/DMβCD
25	980	880	1000	1500
30	950	920	870	1350
37	900	800	800	1180
45	790	810	730	740

The stability constants (K_C) of the inclusion complexes calculated based on the method of Higuchi and Connors [27] at the individual temperatures are given in **Table. 1**. By considering all stability constants of studied complexes at 25◦C, the highest stability was investigated in ED/DMβCD followed by ED/HPβCD, ED/SBEβCD and ED/βCD with 1500, 1000, 880, 980 M^{-1} respectively. The K_C values can be ranked in the order of ED/DMβCD > ED/HPβCD > ED/SBEβCD > ED/βCD. Since the stability constants seems to be responsible for binding behaviors between guest and host molecule, this data indicates that ED is strongly interacting with the DMβCD rather other βCDs. It was noticed that an increase in temperature reduce the stability of the ED/βCDs complexes except in ED/SBEβCD at 25 and 30◦C.

Table.2 . Thermodynamics values of inclusion complexes derived from Van't Hoff plots (R: gas constant-1.985x 10^{-3}, T: absolute temperature-298K).

Thermodynamic Parameter (kcal/mol)	ED/βCD	ED/SBEβCD	ED/HPβCD	ED/DMβCD
ΔH	-2.9	-3.6	-7.7	-11.3
$T\Delta S$	-2.9	-3.3	-5.9	-8.9
$\Delta G_{bind,\,exp}$	0.0	-0.3	-1.8	-2.3
$\Delta G_{bind,\,MM/GBSA}$	0.11 ± 0.51	-1.61 ± 0.92	-3.81 ± 0.65	

Using a Van't Hoff plot equation, the thermodynamic parameters: enthalpy (Δ*H*) *and entropy* (Δ*S*) were calculated by plotting stability constant against with the reciprocal value of temperature. Data was depicted in **Table. 2**. The Gibbs free energy in relation with enthalpy and entropy terms was also calculated. The negative enthalpy energy of −1.8, −3.5, −4.2, and −6.9 kcal/mol for ED/βCD, ED/SBEβCD, ED/HPβCD, and ED/DMβCD inclusion complexes suggested the complex formation is an enthalpy-driven exothermic process. Moreover, the negative value of $\Delta G_{bind,\,\mathrm{exp}}$ indicates the formation of inclusion complex was spontaneous under energetically favorable condition. With regards to the entropy terms, the negative entropy values were seen in all systems suggesting that this complex is entropically unfavorable, but the reaction was compensated by heat. From the Gibbs free binding energy of the host and guest molecule, it can be concluded that all inclusion complexes are spontaneously formed with negative $\Delta G_{bind,exp}$ values with -1.1 ± 0.6 (ED/DMβCD) > -0.7 ± 0.3 (ED/HPβCD) > -0.5 ± 0.3 (ED/SBEβCD and ED/βCD), respectively. The MM/GBSA energy values by molecular dynamics simulations of studied complexes [107] were previously reported and shown in the **Table. 2**.

3.2. Determination of solubility

The aqueous solubility of ED and its inclusion complexes was then studied, and the data shows that the solubility of ED was dramatically increased up to 1.90 times in ED/βCD, 2.12 times in ED/SBEβCD, 6 times in ED/HPβCD, and 34 times in ED/DMβCD complexes respectively. The data is described in **Table 3**.

Table.3 . Solubility of ED and its inclusion complexes at 30ºC

	Solubility of ED (μg/mL)
ED	3.3
ED/βCD	6.3
ED/SBEβCD	7.1
ED/HPβCD	19.9
ED/DMβCD	113.9

3.2. Inclusion complex characterization

3.2.1. Surface morphological changes upon inclusion complexation

Several studies reported that the surface morphological changes of the resulting products were induced upon inclusion complex formation. The SEM images at 1.0 K.X magnification of the studied inclusion complex together with the respective unbound forms are summarized in **Fig. 3**. In case of ED, βCD, and DMβCD pure forms, ED can be defined as the irregular shaped crystals while βCD, and DMβCD are defined as the big and small rod-shaped structures respectively which is in accord with the previous studies [29]. From **Fig. 3**, it can be clearly seen that HPβCD possess the pores on its rugged surface [13] whereas SBEβCD is identified as the small round-shaped feature [22].

Upon molecular complexation, the structures of ED and βCDs are altered into the different morphology. In case of ED/βCD, the bulky rod-like structure of βCD completely disappeared and adopted to the small, fragmented structures. Similarly, ED/HPβCD inclusion complex transformed to a newly formed flake-like or blocky structure without the ragged surface with the spherical cavities of HPβCD and the

irregular shape of ED. ED/SBEβCD completely changed to a new structure discarding the small round shaped structure of SBEβCD and the shape of ED, while ED/DMβCD converted to a newly formed structure leaving the structures of unbound forms.

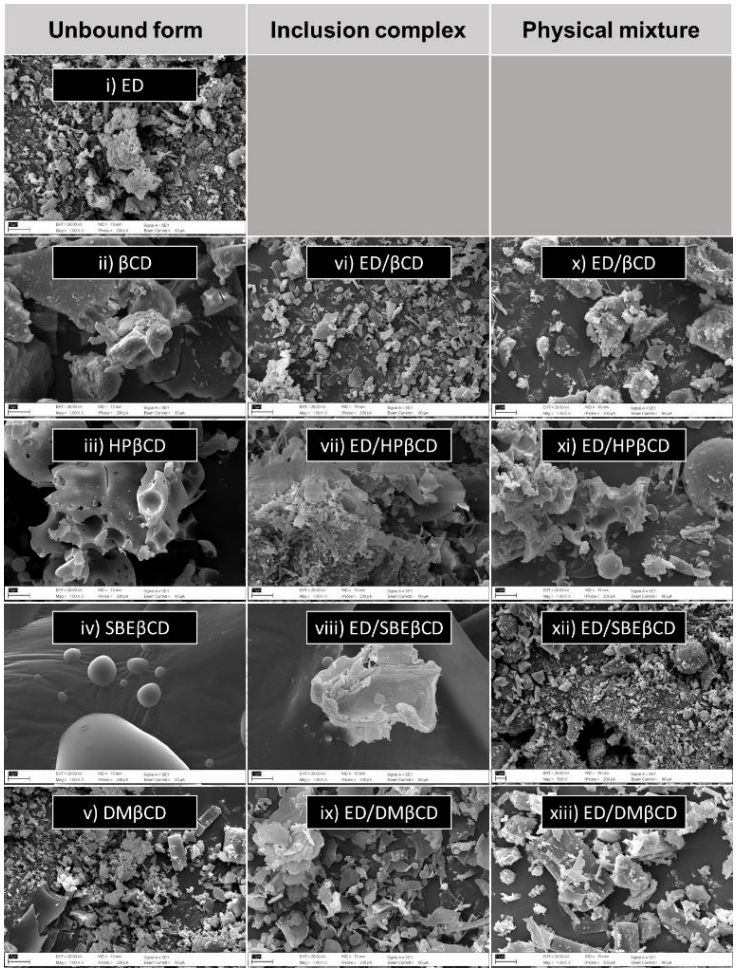


Fig. 3. The SEM micrographs of (i-v) unbound form of ED, βCD, HPβCD, SBEβCD, and DMβCD, (vi-ix) inclusion complex and (x-xiii) physical mixture of ED/βCD, ED/HPβCD, ED/SBEβCD, and ED/DMβCD, respectively, at 1.0 K.X magnification.

3.2.2. Thermal behavior upon inclusion complex formation

Fig. 4. The DSC diagrams of (A) ED, (B) βCD (C) HPβCD (D) SBEβCD (E) DMβCD, (F) ED/βCD, (G) ED/HPβCD (H) ED/SBEβCD, and (I) ED/DMβCD.

3.3. Cytotoxicity screening

To study the potent cytotoxic effect of ED and its inclusion complexes, MTT assay was performed toward KKU-213A and KKU-213B cholangiocarcinoma cells. Cells were firstly treated with logarithmic concentration of ED and its inclusion complexes. The cell viability of ED and its inclusion complexes showed the potent inhibition toward KKU-213A and KKU-213B in concentration-dependent manner. The resulting data is illustrated in **Fig. 5(A)**. It was noted that ED showed the potent cytotoxicity with IC_{50} value of 80.16 ± 4.65 and 49.82 ± 2.25 μM whereas the investigated IC_{50} of studied inclusion complexes were 78.62 ± 2.80 and 57.78 ± 3.96 μM (ED/βCD), 57.94 ± 4.36 and 47.66 ± 1.40 μM (ED/HPβCD), and 52.78 ± 1.57 and 47.00 ± 0.58 μM (ED/DMβCD), respectively to KKU-213A and KKU-213B. The resulting IC_{50} values are plotted in **Fig. 5(B)**. The cytotoxicity screening of unbound forms of βCDs were also investigated and shown in **Fig. 4(C)**. The inhibitory effect was selectively higher in KKU-213A cell line compared to KKU-213B. From MTT assay, ED/DMβCD shows the most potent inhibition toward KKU-213A and KKU-213B.

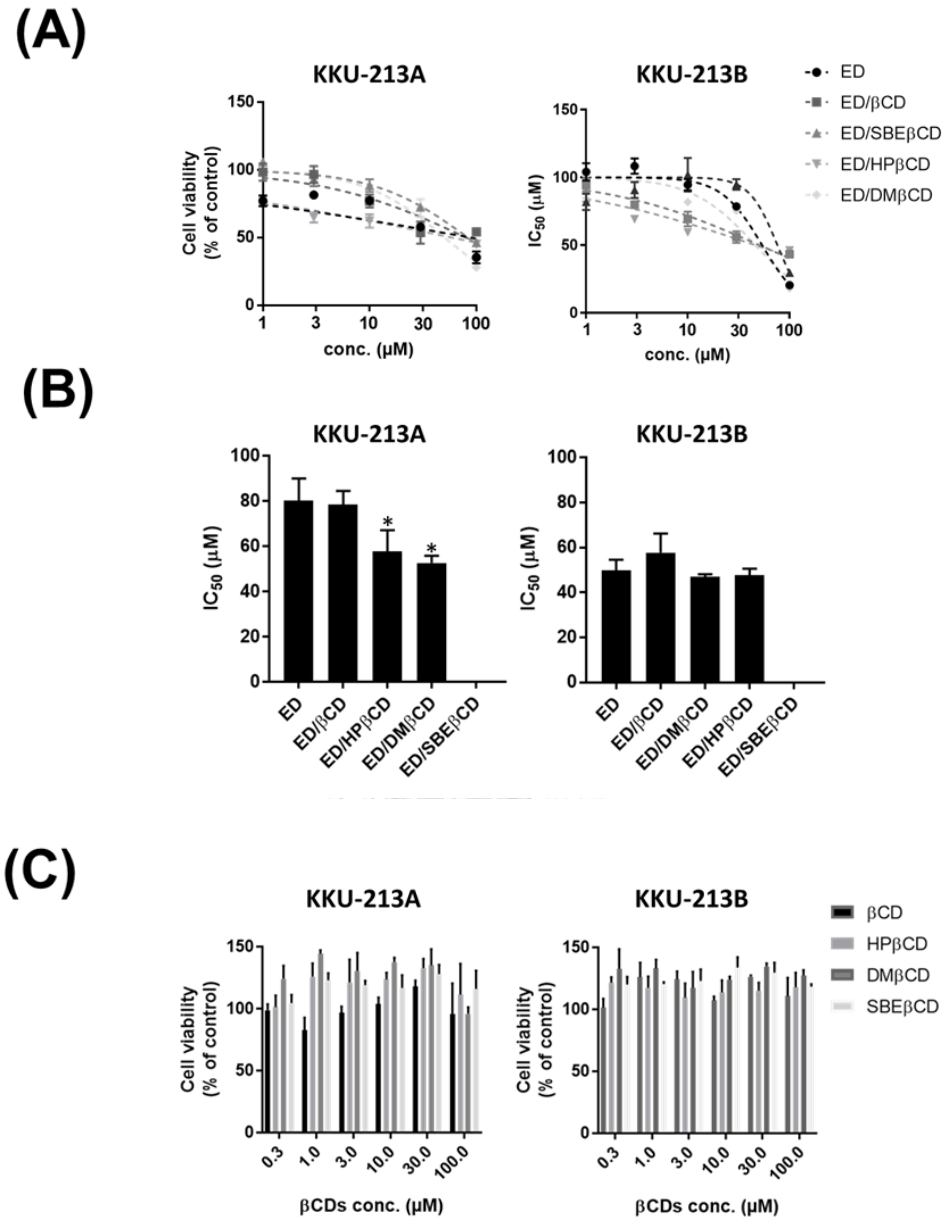


Fig. 4. The cell viability of KKU-213A and KKU-213B cholangiocarcinoma cell lines after treatment with ED, and its inclusion complexes in logarithmic concentration (1, 3, 10, 30, 100μM) for 48h. (B) The IC_{50}(μM) of all investigated compounds (C) Cell viability of KKU-213A and KKU-213B cell lines after treatment with free forms of βCD, HPβCD, DMβCD, and SBEβCD.

4. Conclusion

In vitro studies on inclusion complexation of emodin with various βCDs derivatives were performed. The phase solubility diagrams show the solubility of ED can be enhanced by all studied βCDs and AL-type profile was achieved showing 1:1 interaction between host and guest. The stability constants were decreased when temperature increased. The best stability constant was observed in ED/DMβCD complex at 25ºC. The resulting negative $\Delta G_{bind,\,\mathrm{exp}}$ values indicates that the formation of inclusion complexes was spontaneous and energetically favorable. The inclusion complex characterization by SEM supports the fact that the complex formation induces the surface morphological changes. The water solubility of ED was 3.31 µg/mL at 30 ºC and it can be enhanced dramatically by complexation with parent βCD (1.9 folds), SBEβCD (2.12 folds), HPβCD (6 folds) and DMβCD (34 folds), respectively. The cytotoxicity screening of all studied complexes toward cholangiocarcinoma cell line indicates that inclusion complexation with βCDs can enhance the biological properties of guest molecules by improving its water solubility. Altogether, ED/DMβCD complex shows the best stability, the highest water solubility, and the most promising inhibition pattern toward cholangiocarcinoma cell lines.

CRediT authorship contribution statement

Amy Oo: Writing - original draft, **Khanittha Kerdpol, Ponsiri Liangsakul, Vichai Reutrakul, Sariyarach Thanasansurapong, Siyaporn Putthisen:** Writing - review

& editing, **Kuakarun Krusong, Chutima Kuhakarn, Phornphimon Maitarad, Peter Wolschann, Atit Silsirivanit:** Conceptualization, Writing - review & editing, **Panupong Mahalapbutr:** Conceptualization, Writing - review & editing, Supervision, **Thanyada Rungrotmongkol:** Conceptualization, Writing - review & editing, Supervision. All authors discussed and contributed to the final manuscript.

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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