Preparation and Study of Anticancer Activity of Curcuminoid-Rich *Curcuma longa* Extracts (CRE) and CRE-Cyclodextrin Inclusion Complexes

Likit Lateh

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Pharmaceutical Sciences

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การเตรียมและศึกษาฤทธิ์ต้านมะเร็ งของสารสกัดขมิ้นชันที่มีเคอร์คู มินอยด์ปริมาณสูง (CRE) และการเตรียม CRE-cyclodexdrin inclusion complexes

ผู้เขียน นายลิขิต ลาเต๊ะ

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บทคัดย่อ

สารสกัดขมิ้นชันมี สารเคอร์คูมินอยด์เป็นองค์ประกอบหลัก 3 ชนิด ได้แก่ เคอร์คูมิน, ดีเมทอกซีเคอร์คูมิน และ บิสดีเมทอกซีเคอร์คูมิน ซึ่งมีฤทธิ์ทางเภสัชวิทยาที่หลากหลาย เช่น ฤทธิ์ต้านนุมูลอิสระ ต้านการอักเสบ และต้านมะเร็ง มีรายงานว่าตัวทำละลายที่เหมาะสมในการสกัดสารเคอร์คูมินอยด์ คืออะซิโตนและเอทิลอะซิเตต แต่ตัวทำละลายทั้งสองมีความเป็นพิษต่อผู้ใช้และมีราคาแพง การศึกษานี้จึงมีวัตถุประสงค์ในการเตรียมสารสกัด ขมิ้นชันให้มีสาร เคอร์คูมินอยด์ปริมาณสูง โดยใช้วิธีการสกัดด้วยคลื่นไมโครเวฟซึ่งเป็นวิธีการสกัด ที่เป็นมิตรต่อสิ่ งแวดล้อม และใช้ตัวทำละลายที่มีความปลอดภัย จากการศึกษา ทำให้ได้ สภาวะ การสกัด ที่เหมาะสม ด้วยเครื่องไมโครเวฟ โดยใช้เอทานอลเป็นตัวทำละลายในการสกัด ใช้กำลังไฟฟ้าของเครื่องไมโครเวฟ 900 วัตต์ เปิดเครื่องเพื่อให้คลื่นไมโครเวฟ 3 รอบ โดยในหนึ่งรอบเปิดเครื่อง 3 นาที และปิดเครื่อง 30 วินาที ที่อุณหภูมิ 75 องศาเซลเซียส แล้วนำสารสกัดที่ได้มาแยกให้บริสุทธิ์ขึ้นโดยใช้คอลัมน์ที่บรรจุแมคโครพอรัสเรซิน ชนิด Diaion® HP-20 และใช้สารละลายเอทานอลในน้ำ ร้อยละ 55 และร้อยละ 60 โดยปริมาตร เป็นตัวทำละลายในการชะสาร ตามลำดับ จะได้สารสกัดขมิ้นชันที่สารเคอร์คูมินอยด์ปริมาณสูง โดยมีสารเคอร์คูมินอยด์รวมไม่น้อยกว่าร้อยละ 88 โดยน้ำหนัก

การศึกษาฤทธิ์ต้านมะเร็ง ต่อเซลล์มะเร็ง 4 ชนิดได้แก่ มะเร็งเต้านม (MCF-7), มะเร็งปอด (A-549), มะเร็งลำไส้ใหญ่ (HT-29) และมะเร็งปากมดลูก (HeLa) พบว่าสารสกัดขมิ้นชันที่ มีสารเคอร์คูมินอยด์ปริมาณสูงมีฤทธิ์ต้านมะเร็งดีกว่าสารสกัด หยาบขมิ้นชันด้วยเอทานอล โดยมีค่า IC_{50} เท่ากับ 5.18, 3.46, 2.73 และ 7.66 ไมโครกรัมต่อมิลลิตรตามลำดับ และมีฤทธิ์ที่ดีเกือบเทียบเท่าฤทธิ์ต้านมะเร็งของ สารเคอร์คู มินอยด์บริสุทธิ์ ดังนั้นสำหรับในอุตสาหก รรม การใช้ สารสกัดขมิ้นชัน ในรูปแบบ สารสกัดที่มีเคอร์ คูมินอยด์ปริมาณสูง นี้จึงมีข้อ ดีกว่าการใช้ สารเคอร์คู มินอยด์บริสุทธิ์ เนื่องจากมีขั้นตอนในการเตรียม ที่ง่ายกว่า ทำให้ลดต้นทุนในการผลิต และ ยังเตรียมด้วยกรรมวิธีเป็นมิตรต่อสิ่งแวดล้อมด้วย

การเพิ่มการละลายของสารเคอร์คู มินอยด์โดยการเตรียมในรูปแบบสารประกอบเชิงซ้อนทุติยภูมิและตติยภูมิกับสารไฮดรอกซีโพรพิล -เบต้า- ไซโคลเด็กซ์ตริน และ พอลิไวนิลไพโรลิโดน เค 30 ด้วยวิธี solvent evaporation และนำไปศึกษาคุณลักษณะทางเคมีกายภาพ ด้วย Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), X-ray powder diffraction (XRD) และ scanning electron microscopy (SEM), อัตราการละลาย , ความคงตัว และฤทธิ์ต้านมะเร็ง พบ ว่าสภาวะที่ดีที่สุด สำหรับการเตรียมสารประกอบเชิงซ้อนทุติยภูมิและตติยภูมิ คือ ใช้อัตราส่วนโมลาร์ระหว่างสารสกัดเคอร์คูมินอยด์ปริมาณสูง กับ สารไฮดรอกซีโพรพิล -เบต้า-ไซโคลเด็กซ์ตริน เท่ากับ 1:1 และใช้สาร พอลิไวนิลไพโรลิโดน เค 30 ร้อยละ 9 โดยน้ำหนัก ตามลำดับ สารประกอบเชิงซ้อนทุติยภูมิและตติยภูมิ ที่เตรียมได้ด้วยกรรมวิธีดังกล่าว มีคุณลักษณะทางเคมีกายภาพที่จำเพาะในการเกิดสารประกอบเชิงซ้อนเมื่อเปรียบเทียบกับการเตรียมสารโดยการผสมทางกายภาพ และสามารถเพิ่มการละลายน้ำ ของสารเคอร์คูมิน อยด์ได้เท่ากับ 50 และ 70 ไมโครกรัมต่อมิลลิตร ตามลำดับ จากการศึกษาความคงตัวของสารสกัดพบว่าสาร สกัดเคอร์คู มินอยด์ปริมาณสูง และสารประกอบเชิงซ้อน ทั้งสองชนิดมีความคงตัวเมื่อเก็บใน ภาชนะที่ปิดสนิทและป้องกันแสง ที่อุณหภูมิ 4 - 25 องศาเซลเซียส และในสภาวะเร่งที่อุณหภูมิ 45 องศาเซลเซียส ความชื้น 75% เป็นระยะเวลาอย่างน้อย 4 เดือน นอกจากนี้ ยังพบว่า สาร ประกอบ สารประกอบเชิงซ้อนทุติยภูมิและตติยภูมิมีความเป็นพิษต่อเซลล์มะเร็งเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับสกัดเคอร์คูมินอยด์ปริมาณสูง ดังนั้น สารประกอบเชิงซ้อนทุติยภูมิและตติยภูมิของสกัดเคอร์คูมินอยด์จึงมีศักยภาพที่จะนำไปพัฒนาเป็นผลิตภัณฑ์ยาและอาหารเสริมในระดับอุตสาหกรรมต่อไป

Thesis Title Preparation and Study of Anticancer Activity of Curcuminoid-Rich *Curcuma longa* Extracts (CRE) and CRE-Cyclodextrin Inclusion Complexes

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Major Program Pharmaceutical Sciences

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ABSTRACT

Curcuma longa extracts contain three major curcuminoids, namely curcumin, demethoxycurcumin, and bisdemthoxycurcumin, which possess various pharmacological activities, such as antioxidative, anti-inflammatory and anticancer activities. It has been reported that acetone and ethyl acetate were the most suitable solvents for extraction of curcuminoids. However, these solvents are dangerous and expensive. The present study therefore focused on preparation of curcuminoid-rich *C. longa* extracts (CRE) using a microwave-assisted extraction (MAE) and bio-solvents that have been approved as a green extraction. The optimal conditions of MAE were: employing ethanol as solvent, microwave power of 900 W, with three irradiation cycles (one cycle was 3 min power-on, and 30 sec power-off), at 75°C. The curcuminoid extract was subsequently fractionated on a macroporous resins (Diaion® HP-20) column eluted with 55% and 60% v/v ethanol, respectively, to obtain the extracts enriched in curcuminoids, which contain total curcuminoids of not less than 88% w/w.

CRE exhibited stronger anticancer activity against A-549, MCF-7, HT-29 and HeLa cell lines with the IC_{50} values 5.18, 3.46, 2.73 and 7.66 µg/mL, respectively than that of the crude ethanol extract, and almost equal to that of the pure curcuminoids. Therefore, for industrial applications, a large-scale one-step preparation of CRE has more advantages than the use of pure curcuminoids in terms of convenience, a low-cost production and environmental-friendly process.

Water solubility of curcuminoids in CRE was enhanced by a formulation of binary (CRE/HP-β-CD) and ternary inclusion complexes (CRE/HP-β-CD/PVP K30) with hydroxypropyl-β-cyclodextrin (HP-β-CD) and polyvinylpyrrolidone K30 (PVP K30) using the solvent evaporation method. The physicochemical characteristic of inclusion complexes were investigated by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry

(DSC), powder X-ray diffractometry (PXRD), scanning electron microscopy (SEM) compared with their physical mixture. Dissolution, anticancer activity and stability of the inclusion complexes were also investigated. The optimal conditions for formulation of binary inclusion complex were: employing 1:1 mole ratio between CRE and HP-β-CD and 9% w/w PVP K30, respectively. All the characteristic information demonstrated that the formation of binary and ternary inclusion complexes exhibited specific spectroscopic features and properties. The binary and ternary inclusion complexes improved water solubility of curcuminoids up to 50 and 70 µg/mL, respectively. The CRE inclusion complexes were stable for 4 months when kept in a well-closed container protected from light when stored at 4 - 25°C and accelerated conditions at 45°C, 75% humidity. Both inclusion complexes exhibited stronger cytotoxic activity against the cancer cells than CRE. The inclusion complexes of curcuminoids are therefore potentially applied as pharmaceutical and dietary supplement products.

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I am very happy if a part of my thesis will be useful for further studies.

Likit Lateh

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
/	Per
µg	Microgram (s)
µL	Microliter (s)
µm	Micrometer (s)
µM	Micromolar
AH	Ambient humidity
ANOVA	Analysis
cm	Centimeter (s)
e.g.	For example
et al.	et alia, and others
etc.	et cetera, and other things
g	Gram (s)
GAP	Good agricultural practices
GHz	Gigahertz
h	Hour (s)
HPLC	High-performance liquid chromatography
IC_{50}	The inhibitory concentration at 50% of the tested subject
LD_{50}	The dose of a chemical which kills 50% of a sample population
kg	Kilogram (s)
L	Liters
LC_{50}	The lethal concentration at 50% of the tested subject
MAE	Microwave-assisted extraction
No.	Number
NOEL/NOLE	No observed effect level
nm	Nanometer

LIST OF ABBREVIATIONS (CONTINUED)

mg	Milligram (s)
MHz	Megahertz
mL	Milliliter (s)
mm	Millimeter (s)
mM	Millimolar
PDA	Photodiode array detector
pH	The negative logarithm of the hydrogen ion concentration
ppm	Part per million
CRE	Curcuminoids-rich *Curcuma longa* extract
s	Second (s)
SD	Standard derivation
SEM	Scanning electron micrographs
TLC	Thin layer chromatography
v/v	Volume by volume
W	Watt
w/w	Weight by weight
w/v	Weight by volume

CHAPTER 1

INTRODUCTION

1.1 General introduction

The incidence of mortality and popularity from major types of cancer, at national level, for 184 countries of the world has been published by the International Agency for Research on Cancer. The data information from that research revealed that there are many cancer cases in 2012 worldwide includes cancer death, new cancer cases and people survival with cancer (within 5 years of estimation). The number of those were 8.3 million, 14.1 million and 32.6 million respectively. Besides, it was also stated that the number of cancer cases in 2030 will be increased. The number will be 26 million new cancer cases and 17 million cancer deaths per year. Although many amounts of time and efforts have been spent, cancer remains an offensive killer worldwide (Solowey et al., 2014). The National Cancer Institute Thailand (NCL) has presented that colorectal cancer is the most common cancer in man, followed by lung cancer and liver cancer whereas in women, breast cancer is the most common, followed by cervical cancer and colorectal cancer.

During the last decade, various novel synthetic chemotherapeutic agents that presently being used clinically during the last decade have not succeeded in fulfilling expectations despite the considerable cost of their development. Thus, natural products have received increasing attention for their potential as novel cancer preventive and therapeutic agents. Likewise, many evidences for the potential plant-derived compounds as inhibitors of various stages of tumorigenesis and inflammation-associated processes have underlined the importance of these products in cancer prevention and therapy (Murali et al., 2012; Solowey et al., 2014).

Turmeric (*Curcuma longa* L., family of Zingiberaceae) has been commonly used as spice and medicine, particularly in Asian countries. In Ayurveda medicine, turmeric is primarily used as a treatment for inflammatory conditions. In traditional Chinese medicine, it is used as stimulant, carminative, emenagogue, astringent, detergent, and diuretic (Li et al., 2011). In

Thailand, *C. longa* is an herb reccommennded as a fundamental drug in the primary health care system for treatment of dyspepsia and peptic ulcer (คณะเภสัชศาสตร์มหาวิทยาลัยสงขลานครินทร์, 2551).

Three major curcuminoids, including curcumin, demethoxycurcumin, and bis-demethoxycurcumin have been isolated from *C. longa* (Revathy et al., 2011). These curcuminoids possess anti-cancer, antioxidant, anti-inflammatory, anti-mutagenic, anti-fungal, and antiviral activities (Aggarwal et al., 2003). To dates, there are many studies, both *in vitro* and *in vivo*, indicated the anti-cancer or cancer prevention activity of curcuminoids, especially curcumin (Kuttanet et al., 1985; Simon et al., 1998; Cheng et al., 2001; Hour et al., 2002; Shishodia et al., 2006; Ohori et al., 2006; Hatcher et al., 2008). Curcuminoids or *C. long* extracts are therefore considered as the promising natural occurring cancer preventing agents.

It has been reported that ethanol was the extraction solvent that gave the highest yield of *C. longa* extracts, but their curcuminoid contents were very low, due to containing of oleoresins and other nonvolatile oils. Generally, the use of a pure natural compound is a limitation for industrial application, because its purification process requires many steps, time consuming and high cost (Li et al., 2009). Recent trends in extraction techniques have largely focused on finding the method that could minimize the use of solvents and energy, reduce steps of production, and give a high quality herbal extract. We are therefore interested in preparation of the curcuminoid-rich *C. longa* extracts (CRE) using the green extraction concepts.

There are many preclinical and clinical studies demonstrated a low bioavailability of curcuminoids due to its very low water-solubility (Yallapu et al., 2012). It has been reported that the inclusion complexes, which encapsulated curcumin in the internal cavity of cyclodextrins (CD) increased water-solubility and dissolution rate of curcumin (Mohan et al., 2012). Hydroxypropyl-β-cyclodextrin (HP-β-CD) is much more water-soluble than the natural β-CD and considered as a non-toxic at low to moderate oral and intravenous doses. It can be found in several marketed drug formulations (Brewster et al., 2012). It has been reported that the curcumin/HP-β-CD inclusion complexes can enhance solubility, stability, dissolution rate and bioavailability of curcumin (Ouyang et al., 2012; Jantarat et al., 2014; Yadav et al., 2009). However, due to the relatively high molecular weight of CD, a large amount of CD used in the

formulation limited its application into a convenient and cost-effective dosage form (Anwer et al., 2014). In addition, a ternary complex between drug, CD and water-soluble polymer can reduce the dose of CD and increase the complexation and solubilizing efficiency (Wang et al., 2013). Moreover, the addition of small amounts of polyvinylpyrrolidone (PVP), a water-soluble polymer, to a naproxen/H-β-CD system has improved the complexing and solubilizing efficiencies of HP-β-CD (Mura et al., 2001).

We therefore interested in developing an alternative green extraction method for preparation of CRE. The present study, some alternative green solvents, i.e. propylene glycol (PG), polyethylene glycol 400 (PEG 400), glycerin and ethyl alcohol were determined as the extracting solvent. In addition, a microwave-assisted extraction (MAE) was used as a green extraction method for curcuminoids. The present study focused on a preparation of CRE using a green extraction and fractionation methods as well as improve water-solubility of CRE by formulation of CRE-HP-β-CD inclusion complexes.

1.1 Objectives

The objectives of this study were as follows:

1.1.1 To prepare CRE using the green extraction and fractionation methods

1.1.2 To improve water-solubility of CRE by formulation of the CRE-HP-β-CD inclusion complexes

1.1.3 To characterize the CRE-HP-β-CD inclusion complexes

1.1.4 To determine stability of the CRE-HP-β-CD inclusion complexes

1.1.5 To evaluate *in vitro* anticancer activity of the CRE-HP-β-CD inclusion complexes compared with CRE and the three marker curcuminoids

CHAPTER 2

LITERATURE REVIEW

2.1 Botanical description of *Curcuma longa* L.

Scientific name: *Curcuma longa* L.

Family name: Zingiberaceae

Synonym: *Curcuma domestica* Val.

Common name: ขมิ้นชัน

Figure 2-1 *Curcuma longa* L.

Curcuma longa L. belongs to the Zingiberaceae family. It is a perennial herb that measures up to 1 m height with a short stem (Figure 2-1), distributed throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China. *C. longa* has been well studied due to its economic importance. Its rhizomes are oblong, ovate, pyriform, and often short-branched and used as a household remedy in Nepal and other oriental countries.

2.2 Chemical constituents of *Curcuma longa* L. rhizome

For the phytochemical studies, many compounds were isolated from the rhizomes of *C. longa*, such as monoterpenoids, sesquiterpenoids, steroids, fatty acids diarylheptanoids, diarylpentanoids. The lists of the compounds found in *C. longa* are summarized in Table 2-1.

Table 2-1 Chemical constituents of *C. longa* rhizome.

Chemical groups	Compounds	References
Monoterpenoids	α-pinene, β-pinene, limonene, sabinene, menthofuran, γ-terpinene, ρ-cymene, terpinolene, α-terpinene, myrcene, terpinene-4-ol, α-thujene, ocimene, thymol, camphor, teresantalol, borneol, bornyl acetate, (*E*)-chrysanthenyl acetate, (*Z*)-cinerone, piperitone epoxide, (*Z*)-sabinol, camphene, 3-carene, 2-carene, ascaridole, cineole, *cis*-ocimene, citronellal, geranial, neral, *R*-citronellene, citronellyl pentanoate, nerol, geranyl acetate, *trans*-ocimene, 3-bornanone, iso-artemisia ketone.	Gopalan et al., 2000; Leela et al., 2002; Chowdhury et al., 2008; Usman et al., 2009.
Sesquiterpenoids	(*E*)-sesquisabinene hydrate, γ-gurjunen epoxide, 1-epi-cubenol, cubebene, 7-epi-sesquithujene, caryophyllene, 6α-hydroxycurcumanolide A, curcumanolide A, curcumanolide B, curcumin L, α-humulene, adoxal, (*E*,*E*)-β-farnesene, (*E*,*E*)-α-farnesene, nerolidal nerolidyl propionate, (*Z*)-β-farnesene, *Ar*-turmerone, turmeronol A, urmeronol B	Li et al., 2009; Usman et al., 2009;
Fatty acids	linoleic acid, 8,11-Octadecadienoic acid, methyl ester, palmitic acid (n-hexadecanoic acid), oleic acid, stearic acid	Ma et al., 2006

Table 2-1 Chemical constituents of *C. longa* rhizome (continued)

Chemical groups	Compounds	References
Steroids	β-sitosterol, stigmasterol, gitoxigenin, 20-oxopregn-16-en-12-yl acetate	Chowdhury et al., 2008; Chen et al., 2010
Diarylheptanoid	curcumin (Cu I), demethoxycurcumin (Cu II), bisdemethoxycurcumin (Cu III), tetrahydroxycurcumin, cyclocurcumin, - 1-(4-hydroxy-3-methoxyphenyl)-7-(3, 4-dihydroxyphenyl)-1, 6-heptadiene-3, 5dione. - 1-(4-hydroxyphenyl)-7-(3, 4-dihydroxyphenyl)-1, 6-heptadiene-3, 5dione. - 5-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-4,6-heptadiene-3-one. - 5-hydroxyl-1, 7-bis (4-hydroxy-3-methoxyphenyl)-4,6-heptadiene-3-one. - 1, 7-bis (4-hydroxyphenyl)-1-heptene-3,5-dione. - 5-hydroxyl-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-4, 6-heptadiene-3-one. - 3-hydroxy-1, 7-bis-(4-hydroxyphenyl)-6-heptene-1,5-dione. - 1, 5-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-4, 6-heptadiene-3-one. - 1, 5-dihydroxy-1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-4, 6-heptadiene-3-one. - 1, 5-dihydroxy-1, 7-bis (4-hydroxy-3-methoxyphenyl)-4,6-heptadiene-3-one.	Roth et al., 1998; Park et al., 2005; Zeng et al., 2007; Li et al., 2009; Kita et al., 2009; Chen et al., 2010

Table 2-1 Chemical constituents of *C. longa* (continued)

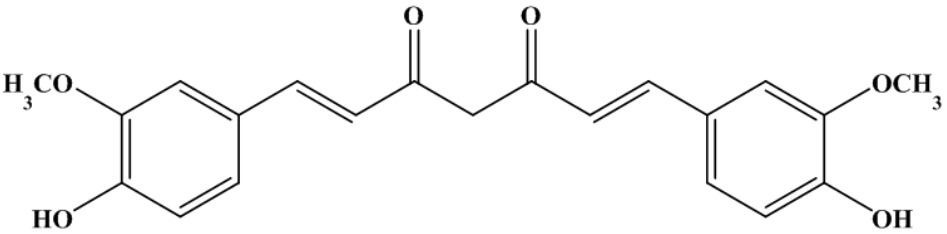
Chemical groups	Compounds	References
Diarylheptanoid	- 1, 5-dihydroxy-1, 7-bis (4-hydroxyphenyl)-4,6-heptadiene-3-one. - 1, 5-epoxy-3-carbonyl-1, 7-bis (4-hydroxyphenyl)-4,6-heptadiene. 1, 7-bis-(4-hydroxyphenyl)-1, 4, 6-heptatrien-3-one.	Roth et al., 1998; Park et al., 2005; Zeng et al., 2007; Li et al., 2009; Kita et al., 2009; Chen et al., 2010
Diarylpentanoids	- 1, 5-bis (4-hydroxyphenyl)-penta-(1E, 4E)-1, 4-dien-3-one. - 1-(4-hydroxy-3-methoxyphenyl)-5-(4-hydroxyphenyl)-1, 4-pentadiene-3-one. - 1, 5-bis (4-hydroxy-3-methoxyphenyl)-penta-(1E, 4E)-1,4-dien-3-one.	Wang et al., 2008

2.3 Chemical structure and properties of curcuminoids

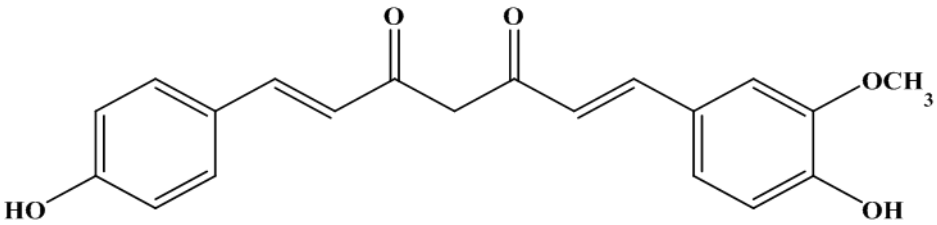
Curcuminoids, a polyphenol, is an active principle of the perennial herb *C. longa* (commonly known as turmeric) (Katsuyama et al., 2009). The yellow-pigmented fraction of turmeric contains curcuminoids, which are chemically related to its principa1 ingredient, curcumin (Cu I). The major curcuminoids present in turmeric are demethoxycurcumin (Cu II), bisdemethoxycurcumin (Cu III) (Fig. 2-2). The major components of commercial curcuminoids are curcumin I (77% w/w), curcumin II (17% w/w), and curcumin III (3% w/w) (Goel et al., 2008). The chemical and physical properties of curcuminoids have been published as summarized in Table 2-2.

Table 2-2 Chemical and physical characteristics of curcuminoids

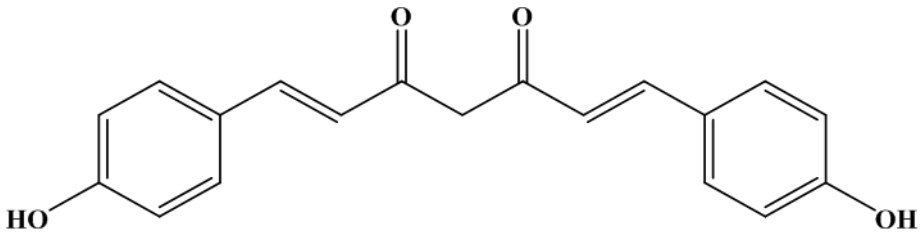
Particulars	Curcuminoids		
	Cu I	Cu II	Cu III
Structure	R1 = R2 = OCH_3	R1 = OCH_3, R2 = H	R1 = R2 = H
IUPAC name	1,7-bis-4-hydroxy-3-methoxyphenyl-hepta-1,6-diene-3,5-dione	1-4-hydroxy-3-methoxyphenyl-7-4-hydroxyphenyl-hepta-1,6-diene-3,5,-dione	1, 7-bis-4-hydroxyphenyl-hepta-1, 6-diene-3, 5-dione.
Chemical formula	$C_{21}H_{20}O_6$	$C_{20}H_{18}O_5$	$C_{19}H_{16}O_4$
MW (g/mol)	368	338	308
pKa	8.54	9.30	10.69



Curcumin (Cu I)



Demethoxyurcumin (Cu II)



Bisdemethoxyurcumin (Cu III)

Figure 2-2 Chemical structures of curcuminoids isolated from *Curcuma longa* rhizome

2.4 Pharmacological activity of curcuminoids

Curcuminoids are main active compounds in *C. longa*, with a variety of pharmacological movements such as antioxidant, anti-inflammatory, anti-nephrotoxicity, antiviral antifungal, anti-arthritic, anti-carcinogenic, anti-Alzheimer's and hepatoprotective properties. The curcuminoids in pharmacological movements have been definitely credited to the curcumin (Cu I). Besides, it is related to two compounding of demethoxycurcumin (Cu II) and bisdemethoxycurcumin (Cu III). The possibilities of curcuminoids in various pharmacological activities were summarized in Table 2-3.

Table 2-3 Pharmacological activities of curcuminoids

Pharmacological activity	Tested Compounds	Model used/study design	Effect/Potency	References
Antioxidant activity	Cu I, Cu II and Cu III	*In vitro* phosphomolybdenum and linoleic acid peroxidation assays.	Phosphomolybdenum: the antioxidant capacity of Cu I (3099 µmol/g), Cu II (2677 µmol/g) and Cu III (2833 µmol/g) and 2677 µmol/g of ascorbic acid equivalents (50 ppm). Linoleic acid peroxidation was found to with Cu I (81.98%), Cu II (81.77%) and Cu III (77%) of butylated hydroxyl toluene equivalents (100 ppm).	Jayaprakasha et al., 2006
	Cu I, Cu II and Cu III	*In vitro* DPPH free radical scavenging and ferric-reducing power (FRAP) assays.	DPPH•$^{+}$ IC_{50} value of Cu I, Cu II and Cu III were 0.72, 6.62 and 80.2 µM respectively. FRAP for Cu I, Cu II and Cu III were1148, 1432 and 667 µM Fe+2/g respectively.	Kalaycıoğlu et al., 2017
	Cu I	*In vitro* β-carotene-linoleic acid assay	Cu I was shown antioxidants (IC_{50} 8.4 µg/mL)	Sökmen & Khan 2016
	Cu I	*In vitro* ABTS radical cation assay and Ferric-reducing power (FRAP) assay	ABTS•$^{+}$ (IC_{50} = 163.4 µg/mL) FRAP (IC_{50} =32.6 µg/mL)	Mošovská et al., 2016

Table 2-3 Pharmacological activities of curcuminoids (continued)

Pharmacological activity	Tested Compounds	Model used/study design	Effect/Potency	References
Anti-inflammatory	Cu II	*In vitro* LPS-induced, iNOS expression and NO production in macrophages	Cu II was inhibited of calmodulin-dependent protein kinase II and LPS induced inducible nitric oxide synthase expression and nitric oxide production	Kim et al., 2010
	Curcuminoids (Cu I, Cu II and Cu III)	*In vitro* antioxidant and COX-1 and COX-2 inhibition assay	Cu I, Cu II and Cu III were inhibited by liposome peroxidations with 58, 40 and 22 % inhibition respectively (at 100 µg/ml). It were inhibited COX-1 enzyme with 32, 38.5 and 39.2 % inhibition* respectively and also showed inhibition of the COX-2 enzyme with 89.7, 82.5 and 58.9 % inhibition* respectively (*at 125 mg/ml)	Ramsewak et al., 2000
Anti-Alzheimer's	Curcuminoids (Cu I, Cu II and Cu III)	*In vivo* anti-Alzheimer's (Spraguee Dawley male rat)	Cu I, CuII and Cu III were inhibited in the frontal cortex, hippocampus with *ex vivo* AChE assay and effective in memory enhancing effect.	Ahmed et al., 2009; Ahmed et al., 2011
Antiviral activity	Cu I	Madin-Darby canine kidney (MDCK) cells	Cu I was interrupted virus-cell attachment, which leaded to inhibition of virus propagation.	Chen et al.,2010

Table 2-3 Pharmacological activities of curcuminoids (continued)

Pharmacological activity	Tested Compounds	Model used/study design	Effect/Potency	References
Anti-HIV-1 activity	Cu I	*In vitro* HIV-1 integrase inhibition assay	Cu I was inhibited HIV-1 integrase enzyme with IC_{50} 40 μM	Mazumder et al., 1995
Antifungal activity	Cu I and Cu II	*In vitro* antifungal assay (*Candida albicans*)	Cu I was shown antifungal effect stronger than Cu II	Zhang et al.,2012
Hepatoprotective activity	Cu I	*In vivo* hepatoprotective assay (adult Swiss albino mice)	Cu I was induced the release of lactate dehydrogenase and reduced lipid peroxidation	Naik et al., 2004
Anti-arthritic activity	Cu I	*In vivo* anti arthritis assay (female albino Wistar rats)	Cu I was reduced arthritis and alleviated hepatocellular injury caused by methogtrexate.	Banji et al., 2011
Antimutagenic and anticarcinogenic activity	Curcuminoids (Cu I, Cu II and Cu III)	*In vitro* anti mutagenicity assay and *in vivo* anticarcinogenic assay (swiss albino mice model)	Cu III (87%) was most active when compared with Cu II (70%) or Cu I (68%) %inhibition* respectively (at concentration 100μg/plate)	Anto et al., 1996
Anti amoebicidal activity	Cu I	*In vitro* antiamoebicidal assay (*Entamoeba histolytica*)	Cu I was inhibited growth and cell viability of *E. histolytica* (65.5% inhibition at 300 μM)	Rangel-Castañed et al., 2018
Anti-obesity activity	Cu I	*In vitro* LDL stimulated in hepatic stellate cell (HSC)	Cu I was reduced of LDL receptor and cellular cholesterol in activated HSCs.	Kang & Chen, 2009

Table 2-3 Pharmacological activities of curcuminoids (continued)

Pharmacological activity	Tested Compounds	Model used/study design	Effect/Potency	References
Anti-nephrotoxicity	Cu II	Male Wistar-Albino rats	Cu II was decreased in inflammation and apoptosis during histopathological examination	Ahmida, 2012
Antiaging activity	Cu I	*In vitro* glutathione-*S*-transferase (GST) assay and Western blot analysis in human skin fibroblasts (ASF-2 cells)	Cu I was induced cellular stress responses in normal human skin fibroblasts through phosphatidylinositol 3-kinase/Akt pathway and redox signaling	Lima et al., 2011

LPS: lipopolysaccharide; iNOS: inducible nitric oxide synthase; COX: cyclooxygenase; AChE: acetylcholinesterase; HIV-1: human immunodeficiency virus type 1; LDL: low-density lipoprotein

2.5 Anticancer activity of curcuminoids

Curcuminoids have been shown to possess anti-cancer activities via their effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis (Vallianou et al., 2015). curcuminoids has shown anti-proliferative effect in multiple cancers, and is an inhibitor of the transcription factor nuclear factor kappa B (NF-κB) (Leclercq et al., 2004; Olivera et al., 2012) and downstream gene products including epidermal growth factor receptor (EGFR), c-myc, B-cell lymphoma 2 (Bcl-2), cyclooxygenase-2 (COX-2), nitric oxide synthase (NOS), Cyclin D1, tumor necrosis factor alpha (TNF-α), interleukins and matrix metalloproteinases-9 (MMP-9) (Allegra et al., 2017). Moreover, the increasing factor receptors and cell cohesion molecules embraced in tumor growth, angiogenesis and metastasis is one of the varieties effects of curcumins. (Kunnumakkara et al., 2008; Shanmugam et al., 2015). In this review, it is focusing on curcuminoids in *C. longa*, and effects of curcuminoids on the anticancer activity which are summarized in Table2-3 and Figure 2-3. Therefore, curcuminoids compounds were processed anticancer activity through multiple signaling pathways (Figure 2-3). Especially, curcumin has shown the effect on various cellular pathways of different types of cancer (Figure 2-4).

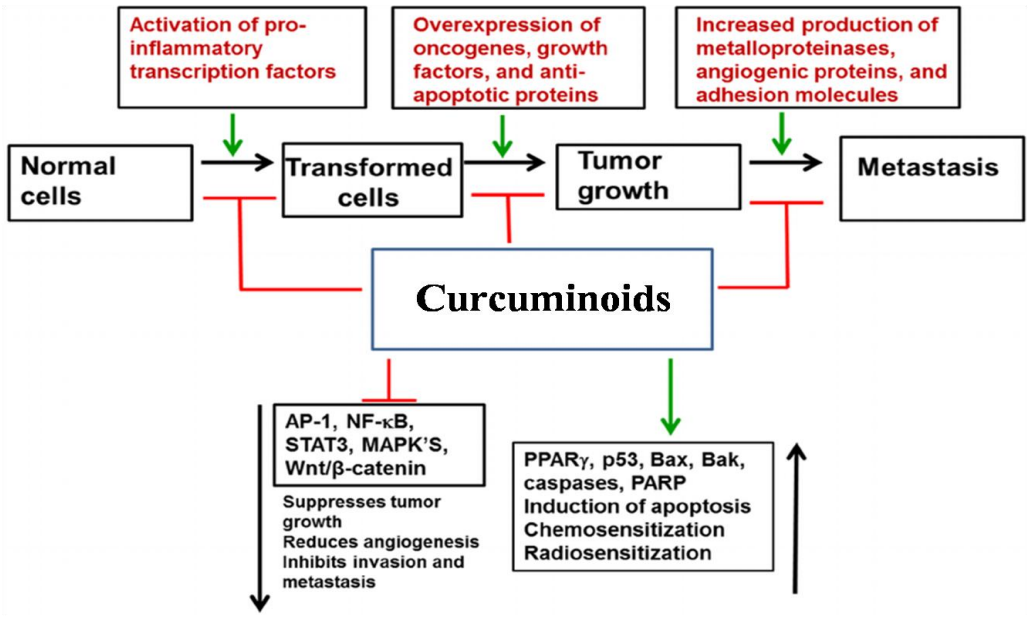


Figure 2-3 Potential anti-cancer functions of curcuminoids in negatively regulation tumor initiation, progression and metastasis (Shanmugam et al., 2015).

Table 2-4 *In vitro* anticancer activity of curcuminoids

Cell type	Tested compound	Effect/Potency	References
Human breast adenocarcinoma cell (MCF-7)	Cu I, CuII and Cu III	Inhibited cell proliferation of MCF-7 cells of human breast. Cu II > Cu I> Cu III	Simon et al., 1998
Cell line of human breast (MCF-7)	Cu I	Influence apoptosis in tumor cells via a p53-dependent route in which Bax is the downstream effector of p53.	Choudhuri et al., 2002
Cell line of human breast (MDA-MB-231)	CuII	Inhibited the DNA binding activity of nuclear factor-kappa B (NF-ĸB), which is known to mediate the expression of matrix metalloproteinase-9 (MMP-9), urokinase plasminogen activator (uPA), uPA receptor (uPAR), intercellular adhesion molecule-1(ICAM-1), and chemokine receptor 4 (CXCR4).	Yodkeeree et al., 2010
Human breast adenocarcinoma cell lines (MDA-MB-231, MDA-MB-435S, and MCF-7)	Curcuminoids	Inhibited cell proliferation and activation of apoptosis in the cell lines by increase in proportion to the cellular uptake of curcuminoids	Chang et al., 2001
Human breast cancer Cell line (MDA-MB-231)	Curcuminoids	Inhibited the growth factor (TGF)-β-stimulated and parathyroid hormone-related protein (PTHrP) secretion (IC_{50} = 24 μM) and decreases in phosphor-Smad2/3 and Ets-1 protein levels in MDA-MB-231 cells.	Wright et al., 2012

Table 2-4 *In vitro* anticancer activities of curcuminoids (continued)

Cell type	Tested compound	Effect/Potency	References
Cell line of human breast (MCF-7)	Cu I	Inhibited the growth of MCF-7 cell by inducing apoptosis in a dose- and time-dependent manner, accompanied by a decrease in MCF-7 cell viability.	Koohpar et al., 2015
Cell line of human breast (MCF-7)	Cu I	Inhibited cell viability and induced cytotoxicity of the cell by downregulated miR-21 expression and upregulating the PTEN/Akt signaling pathway in MCF-7 cells	Wang et al., 2017
Cell line of human breast (MCF-7, T47-D, MDA-MB-468) and human prostate cancer cell lines (PC-3 and LNCap)	Cu I	Induced apoptosis in breast cancer and prostate cancer cells via a p53-dependent pathway.	Choudhuri et al., 2002
Human prostate adenocarcinoma cell line (PcBraA1)	Cu I	Inhibited the proliferation of PcBraA1 cells by induction apoptosis 52% (at 25 μM) and necrosis 98% cell died (at 50 μM)	Piantino et al., 2009
Human prostate cancer cells line (PC-3)	Cu II	Inhibited cell proliferation, migration, invasion, and induced apoptosis in PC-3 cells.	Ni et al., 2012
Hepatic stellate cell line (HSC-T6)	Cu I and Cu II	Induced apoptosis in HSC-T6 cells	Lin et al., 2009

Table 2-4 *In vitro* anticancer activities of curcuminoids (continued)

Cell type	Tested compound	Effect/Potency	References
Human prostate cancer stem cells (HuPCaSCs)	Cu I	Reduced the expression of long non-coding RNA (lncRNA-ROR) and increased miR-145 concentration in cells, where miR-145 prevents cell proliferation by decreasing octamer-binding transcription factor 4 (Oct4) expression	Liu et al., 2017
Human ovarian cancer cell line (Ho-8910)	Cu I	Inhibited the growth and induce apoptosis in cells. As well as a decrease in expression of Bcl-2, Bcl-XL and procaspase-3 and increased level of p53 and Bax in the curcumin-treated cells.	Shi et al., 2006
Human ovarian cancer cell line	Cu I	Inhibited the proliferation of cisplatin-resistant cancer cells through the induction of superoxide generation, G2/M arrest, and apoptosis	Weir et al., 2007
Human ovarian cancer cell line (SKOV-3)	Cu III	Inhibited the growth and induce apoptosis in cells and a decrease in expression of Bcl-2, Bcl-XL and NF-ΚB.	Duan et al., 2011
Human ovarian cancer cell line	Cu I	Inhibited the proliferation and microvessel density and increased in cell apoptosis	Cai et al., 2013
Human hepatocellular liver carcinoma cell line	Cu I	Inhibited the cell proliferation through the activation of caspase-9 and -3 and free radical generation.	Notarbartolo et al., 2005

Table 2-4 *In vitro* anticancer activities of curcuminoids (continued)

Cell type	Tested compound	Effect/Potency	References
Human ovarian cancer cell lines (SKOV-3)	Cu II	Inhibited cellular oxidative stress and subsequently inactivating nuclear factor kappa B (NF-ΚB) pathway in SKOV-3 cells.	Pei et al., 2016
Human lung carcinoma cell line (A549)	Cu I	Reduced metastatic of A549 cells through inhibition of MMP-2 and MMP-9 in mitogen-activated protein kinase kinase kinase 3 (MEKK3), phosphor-extracellular signal-regulated kinase (p-ERK) signaling pathways.	Lin et al., 2009
Human small cell lung cancer cell line (SCLC)	Cu I	Reduced angiogenesis through suppression of the signal transducer and activator of transcription 3 (STAT3) signaling pathway in SCLC cell.	Yang et al., 2012
Human lung cancer cell line (NCI-H460)	Cu II	Induced cell morphological changes and decreased the percentage of viable NCI-H460 cells and induced apoptosis based on the cell distribution in the sub-G1 phase	Ko et al., 2015
Human cervical carcinoma cell line (HeLa)	Cu I	Inhibited the growth of cells through multiple mechanisms including induced apoptosis through up-regulating caspase-3 and down-regulating expression of Bcl-2 and BCL-xl.	Jing et al., 2004
Human cervical carcinoma cell line (HeLa)	Cu I	Inhibited the proliferation of HeLa cells by induction apoptosis	Jing et al., 2007

Table 2-4 *In vitro* anticancer activities of curcuminoids (continued)

Cell type	Tested compound	Effect/Potency	References
Human colon adenocarcinoma (HTC15, HCT116) and and human larynx cancer (Hep-G2) cell lines	Cu I and catechins	Inhibited the proliferation of HCT 15, HCT 116, and Hep-G2 cells by apoptosis pathway.	Manikandan et al., 2012
Human colorectal carcinoma cell line (HCT116)	Cu I and Cu II	Induced rapid DNA double-double stand break-in an HCT116 cell with IC_{50} of Cu II (30 μM) and Cu I (10 μM)	Basile et al., 2013

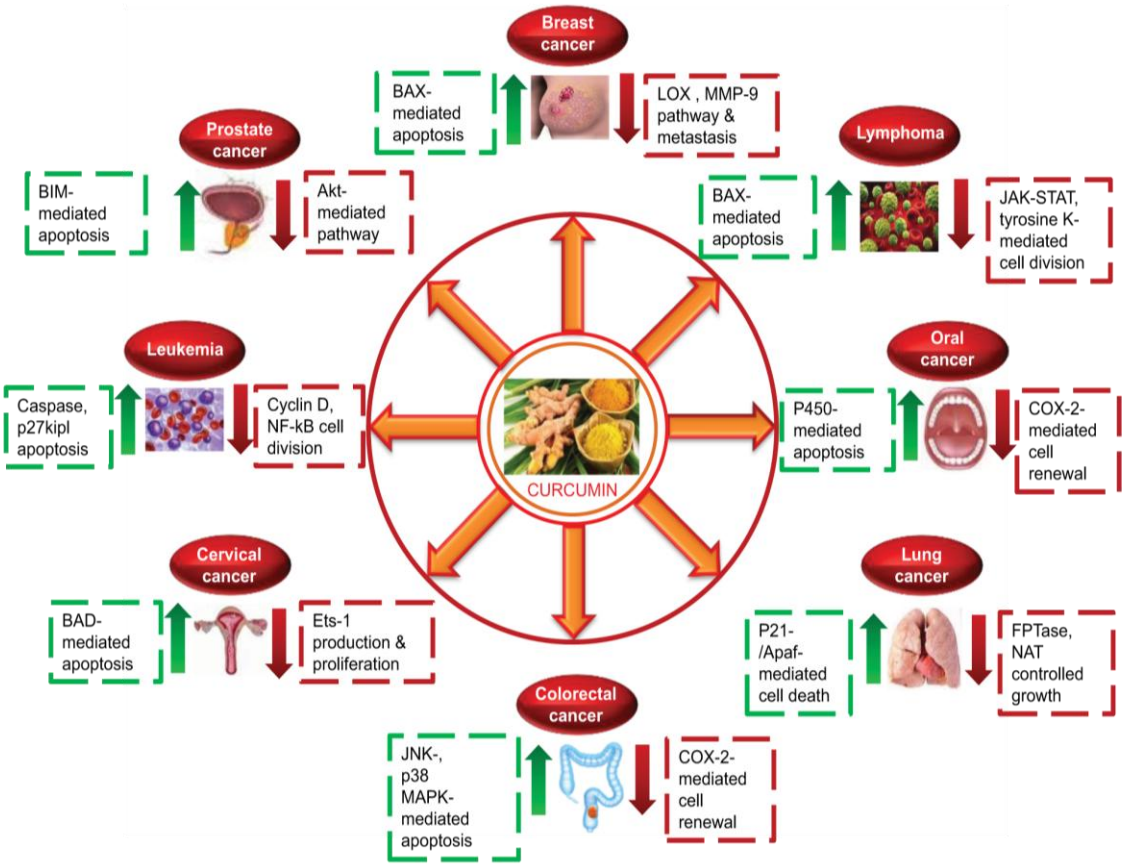


Figure 2-4 Effect of curcumin on various cellular pathways of different types of cancer (Panda et al., 2017)

2.6 Quality and standardization of turmeric products

2.6.1 Marker compounds

The significant curcumin contents as 0.58 to 6.5% on a dry weight basis is depending on the factor of cultivating resources, the harvest ages and drying process in commercial field which made. Nevertheless, the curcuminoids can be rapidly decomposed under certain storage conditions. The chemical variability will result in inconsistent and uncertain efficacy in experiments and clinical trials. Thus, it is neessary for standardization of chemical ingredients in turmeric products.

Regarding to the Thai Herbal Pharmacopoeia recommended that dried turmeric should contain no less than 6% of turmeric oil (v/w) and 5% of total curcuminoids (w/w). While WHO (World Health Organization) is suggesting not less than 4.0% of volatile oil, and not less than 3.0% of curcuminoids in turmeric. In our initial analysis for chemoprevention of colorectal cancer, it is needed that the rhizomes must not contain less than 5% of total contents of three bioactive curcuminoids and the experimental rhizome extracts contain not less than 25% of the three curcuminoids (Aggarwal et al., 2003).

Many customary drugs or their precursors are derived from plants. Nevertheless, it is a difference between contributing a pure isolated chemical and the same chemical in a plant matrix. Whether this "chemical complexity" is advantageous, it is still a matter of some debate. Synergistic effect of compounds is an important concept in medicinal plant use. Chemical complexity has a greater advantage than pure isolated compounds due to reduced costs and less time required for isolation, while pharmacological effects were equal. This needs to be taken into account when considering the preparation of active constituent-rich extracts (Panichayupakaranant *et al*., 2009; Sakunpak et al., 2009; Puttarak et al., 2010 Kaewchoothong et al., 2012).

2.6.2 Conventional extraction methods

The Soxhlet, hydrodistillation, low-pressure solvent extraction and supercritical fluid extraction (SFE) using carbon dioxide (CO_2) with various co-extraction solvents are such different kind of extraction methods that significantly affected by extraction rate, composition of essential oils and curcuminoids. The lowest yield was gained in the hydrodistillation process (2.1%) while the biggest yield (27% weight) was obtained in the Soxhlet extraction with ethanol. Presently, the optimal co-solvent for SFE was a mixture of isopropyl alcohol and ethanol. In basis known, though hydrodistillation and SFE processes might manufacture different yields of total oils, but the percentage of their major compounds extracted out by these two methods present a result in same way.

2.7 Green extraction of the natural product

Green extraction is based on the designs and discovery of extraction procedure, which will relieve energy destruction, allows the use of renewable natural products, alternative solvents

and high-quality extract and ensure a safe (Li et al., 2013; Chemat et al., 2012), following the existing study of the principles Green extraction that is six points as scheduled as a discovery to create novel and green definition sustainable technologies consist (Chemat et al., 2012):

1. The selection factor makes the innovation of alternation and use of rebuildable plant resources
2. Creation of co-goods in place of trash to consist the agro-refining manufactures
3. Decrease section works controlled processes and robust and favor protected
4. Reduce energy consumption by using innovative and energy recovery
5. Aim for a biodegradable extract and non-denatured without contaminants
6. Use of alternative green solvents and agro-solvents or principally water.

2.7.1 Alternative solvents for green extraction

Present rules which have a moving straightway impinge in diminution the use up volatile organic compounds and petrochemical solvents. Productions which consuming organic solvents must be displayed the safety of ingredients as respects to solvent traces to present the absence of risk during extraction. As the fact of majority of solvents in organic types are volatile, combustible or can be poisonous greenhouse effect and the responsible for environmental contamination. Safety, economic and environmental features will be leading to force the production and manufactuers to be back to the greener solvents.

Amongst the solvents of green, the bio- or agro-solvents show a major role in the displacement of solvents of petro-chemistry. They are an alternative resource produced by biomasses such as starch, wood, fruits or veggie oils. These have been in an extreme level of solvent potential, non-poison, biodegradable and also non-combustible. The cause of drawback and restriction definitely is the big volume of viscosity, cost, generation of off-flavors and high boiling point. Table 2-5 summarizes alternative solvents for green extraction. In addition, in the past, the research on the alternative solvents had focused on halogenated hydrocarbon, hydrocarbon, sulfur-containing solvent, oxygenated hydrocarbon solvents. From the previous study a comprehensive review of the alternative solvents explored in the past can be found in the literature (Table 2-6).

Table 2-5 Alternative solvents for green extraction (Chemat et al., 2012)

Solvent	Extraction Technique	Solvent Power			Health & Safety	Environmental Impact
		Polar	Weakly Polar	Non-Polar		
Solvent-free	Microwave hydro-diffusion and gravity (essential oils)	+++	+		+++	+++
	Pulse electric field (antioxidants, pigments)	+++	+		+++	+++
Water	Steam distillation (essential oils)	++	+		+	+
	Microwave-assisted distillation (essential oils)	+++	+++	+	+	++
CO_2	Extraction by sub-critical water (aromas)	+	++		+	+
	Supercritical fluid extraction (decaffeination of tea and coffee)	-	+	+++	+	+
Agro-solvent	Glycerol (polyphenols)	+	+	-	-	+

Positive (+), negative (-): (+/-) = low, (++/--) = medium and (+++/---) = height

Table 2-5 Alternative solvents for green extraction (continued)

Solvent	Extraction Technique	Solvent Power			Health & Safety	Environmental Impact
		Polar	Weakly Polar	Non-Polar		
Agro-solvent	Ethanol (pigments and antioxidants)	+	+	-	-	+
	Terpenes such as *d*-limonene (fats and oils)	-	-	++	-	+
Petro-chemical solvents	*n*-Hexane (fats and oils)	-	+	+++	---	---

Positive (+), negative (-): (+/-) = low, (++/--) = medium and (+++/---) = hight

2.8 Microwave-assisted extraction (MAE)

Microwave-assisted extraction is one of the most important techniques for extracting valuable constituents from herbal medicines. Recently, a large number of papers on the application of MAE using microwave energy for the extraction of active constituents from plant matrix have been published and lots of remarkable results have been achieved. These reports supported the extraction efficiency by MAE to be comparable or higher than conventional and modern extraction methods. The main reasons for enhanced performance, when using MAE over other methods, are a reduction of extraction time, reduction in the consumption of organic solvents, increment of extraction rate and reduction of extraction temperature, energy and cost.

Table 2-6 Examples of alternative solvents for green extraction of target compounds from plants.

Alternative solvents	Commonly solvents	Extraction technique	Target compounds	Plants	References
Limonene	Hexane	Solid-liquid extraction	Oil	Rice bran	Liu *et al.*, 2004

Table 2-6 Examples of alternative solvents for green extraction of target compounds from plants.

Alternative solvents	Commonly solvents	Extraction technique	Target compounds	Plants	References
Limonene	Hexane	Microwave-integrated Soxhlet extraction	Fats and oils	Limonene	Hexane
	Hexane	Soxhlet extraction	Oil	Microalgae	Tanzi et al., 2012
Sunflower oil	Hexane	Ultrasound-assisted extraction	Carotenoid	*Daucus carota* L.	Li et al., 2013
Rapeseed oil, soybean oil, corn oil and olive oil	Ethyl acetate	Super Masscolloider extraction	Curcuminoids	*Curcuma longa*	Takenaka et al., 2013
Polyethylene glycol	Methanol	Microwave-assisted extraction	Flavone, coumarins	*Lysionotus pauciflorus*, *Cortex fraxini*	Zhou et al., 2011
Water	-	Microwave-assisted extraction	Anti-oxidants	Rosemary	Rodriguez et al., 2012
	-	Microwave-assisted extraction	Glycosides	*Stevia rebaudiana*	Jaitak et al., 2009

2.8.1 Theory and principle of MAE

Microwave is electromagnetic radiations with a frequency (0.3 to 300 GHz). In order to avoid interferences with domestic, radio communications and industrial microwaves generally operate at 2.45 GHz. Wherewith to their microwaves possess electric, magnetic fields and

electromagnetic nature which are perpendicular to each other. The electric field causes heating via two simultaneous mechanisms they are dipolar rotation and ionic conductance. Dipolar rotation is due to the alignment on the electric field of the molecules possessing a dipole moment in both the solid sample and the solvent. This vibration produces conflicts with surrounding molecules and thus the discharge of thermal energy into the medium. With a frequency of 2.45 GHz, this spectacle germinates 4.9×10^9 times per second and the effecting heating is very fast. Indeed, the major the dielectric constant of the solvent (Table 2-7), the more optimal the heating. Therefore, difference classical conductive heating methods, microwaves heat the all sample simultaneously (Figure 2-9). In the instance of extraction, the advantage of microwave heating is the detracting of weak hydrogen bonds encourage by the dipole rotation of the molecules. However, a higher viscosity of the medium lowers this mechanism by affecting molecular rotation (Kaufmann and Christen, 2002). In addition, the evacuation of dissolved ions enhances solvent perforation into the matrix and thus encourages the solvation of the analyte. Ionic currents are induced in the solution by the electric field. As the medium opposes these currents, frictions occur and heat is liberated by a Joule effect.

Table 2-7 Dielectric constants and dipole moment values of some commonly used solvents

Solvent	Dielectric constant (20 ºC)	Dipole moment (25 ºC) (Debye)
Hexane	1.89	<0.1
Toluene	2.4	0.36
Dichloromethane	8.9	1.14
Acetone	20.7	2.69
Ethanol	24.3	1.69
Methanol	32.6	2.87
Water	78.5	1.87

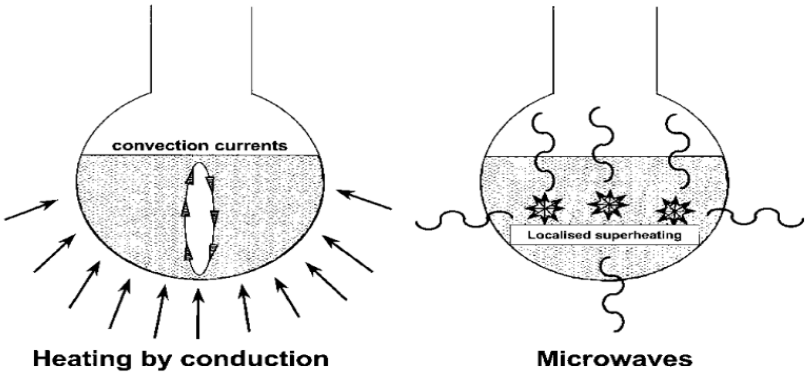


Figure 2-5 scheme of the heating principal by conduction in the classical method of extraction and by microwave irradiation in microwave assisted extraction (Kaufmann and Christen, 2002).

This phenomenon depends on the size and charge of the ions present in the solution. The effect of microwave energy is sturdily dependent on the solid matrix and the nature of both the solvent. Solvents normally used cover a wide range of polarities (heptanes to water). Most of the time, the select solvent procedure a strongly absorbs microwave energy and high dielectric constant, Nevertheless, the extracting selectivity and the potentiality of the medium to interact with microwaves can be regulated by handing mixtures of solvents. Similarity, the matrix itself interacts with microwaves while the surrounding solvent possesses a low dielectric constant and thus remains cold. This latter situation represents some obvious advantages in the case of thermosensitive compounds and has been successfully used for the extraction of essential oils. Really, microwaves interact selectively with the polar molecules show in trichomes, vascular tissues or glands. Localised heating leads to the inflation and break of cell walls and is followed by the release of essential oils into the solvent. This predicament can also be incurred when a dry sample has been re-hydrated before extraction. Truly, moisture content is essential in MAE because water locally superheats and encourages analytes to be liberated into the surrounding medium. Moreover, control of the water content of the matrix allows more reproducible results (Kaufmann and Christen, 2002).

Even though many of the initial publications dealt with natural product extraction, the majority of the applications concern the extraction of pollutants from environmental matrixes. The use of MAE for environmental analysis has newly been thoroughly reviewed, and therefore, only applications to natural products are reported in this review and these are summarized in Table 2-8.

Table 2-8 Application of MAE to natural product extraction

Chemical groups	Target compounds	Plant material	MAE system	Extraction conditions	References
Alkaloids	Berberine, Palmatine, Jatrorrhizine	*Coscinium fenestratum*	Open vessel MAE	Irradiation power, 300 W; extraction time, 15 min and 60 % ethanol as solvent	Deevanhxay et al., 2009
Flavonoids	Silybinin	*Silybum marianum* (seed)	Domestic microwave	Irradiation power, 600 W; extraction time, 8 min and 80 % ethanol as solvent	Dhobi et al., 2009
Phenolic compounds	Phenolic acids	Citrus mandarin (Peels)	Microwave oven	Irradiation power,152 W; extraction time, 49 s and 66% methanol as solvent	Hayat et al., 2009
Polysaccharides	Pectin	*Citrus paradisi* (Peels)	Microwave oven	Irradiation power, 900 W; extraction time, 6 min and water as a solvent	Bagherian et al., 2011
Steroids	Withaferin A	*Withania somnifera* (leaves)	Microwave oven	Irradiation power, 900 W; temperature of 50 °C; extraction time, 2 min and methanol as solvent	Jyothi et al., 2010

Table 2-8 Application of MAE to natural product extraction (continued)

Chemical groups	Target compounds	Plant material	MAE system	Extraction conditions	References
Terpenoids	Pentacyclic triterpenes	*Centella asiatica* (Leaves)	Domestic microwave	Irradiation power, 600 W; temperature,75 °C, four irradiation cycles; four extraction times and ethanol as solvent	Puttarak and Panichayu-pakaranant, 2013
Essential oil	-	Eucalyptus (Leaves)	Domestic modified microwave	Irradiation power, 1000W; extraction time, 3 min and ethanol as solvent	Saoud et al., 2006

The proposed MAE for curcumin presented a drastic lessening in extraction time with much better precision when correlated to conventional extraction methods. The major mechanism answerable for extraction efficiency enhancement was the two heating spectacle of sample matrix and solvent, which worked in capable rupture of plant cell wall. The concurrent heating of the sample and the solvent further enhanced the solubility of curcumin (Mandal *et al*., 2008). The conventional soxhlet assisted extract (SAE) with acetone as solvent of *C. longa* powder resulted in a 2.1% curcumin yield after extraction (8 h). Using the soxhlet extraction as the basis, the curcumin yields of ultrasonic assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical carbon dioxide assisted extraction ($SCCO_2E$) methods were calculated. The MAE of *C. longa* for 5 min improved a maximum of 90 % curcumin. The comparison of yield and the time required for the extraction of curcumin presented that MAE method is rather capable than UAE, SAE and $SCCO_2E$ methods (Wakte *et al*., 2011). The idea can be reasonable to all natural products and if predicated properly, can demonstrate to be a potent tool for large-scale industrial application and sample preparation.

2.9 Limitation of curcuminoids

Reasons for low bioavailability of any compound within the body are related to poor absorption, high rate of metabolism, and rapid elimination and clearance from the body (Setthacheewakul et al., 2010). A major limiting factor of curcumin is its low solubility in both acidic and neutral pH, poor absorption in the gastrointestinal environment, and very low oral bioavailability**.** It has been reported that the pharmacokinetics studies of curcumin have shown low intestinal absorption and rapid metabolism of curcumin toughly curtails its bioavailability. Curcumin is classified as a biopharmaceutical classification system (BCS) class IV molecule on the basis of its poor aqueous solubility (less than 0.6 μg/mL in pure water) and sensible to degradation, especially under alkaline conditions (pH>7) (John et al., 2013). Moreover, in the gastrointestinal tract (GI) tract, only soluble curcumin can be absorbed through luminal epithelial cells. Insoluble curcumin descends in the tract, resulting in excretion with faeces. Therefore, solubilizing curcumin in the GI tract is the first step towards enhanced absorption (Kurita & Makino, 2013). Accordingly, a simple way of solving the limiting factors of curcumin is to improve its solubility various types of solubility improvement techniques can be categorized into physical modification, chemical modifications of the drug substance, and other techniques are summarized in Figure 2-6 (Hani & Shivakumar, 2014). From previous study a comprehensive review of the basic approaches for solubility enhancement of curcumin in the past can be found in the literature.

2.10 Cyclodextrins inclusion complexes

2.10.1 Structure and properties of cyclodextrins

Cyclodextrins (CDs), a cyclic amylase derived from oligomers with a hydrophilic outer surface and a hydrophobic inside cavity, is well known for its potentiality to form an inclusion complex with difference guest molecules. The α-CD, β-CD and γ-CD are the furthest common CDs used as formulation vehicles including of six, seven, and eight D-(+)-glucopyranose parts attached by α-1,4 linkage (Figure 2-7) (Del Valle, 2004). β-CD is the most productive and the lowest price, Nevertheless, its solubility in water is low (approximately 2%) and the toxicity of β-

CD limits its further application in nutraceutical, dietary supplement and pharmaceutical formulations (Qiu et al., 2014).

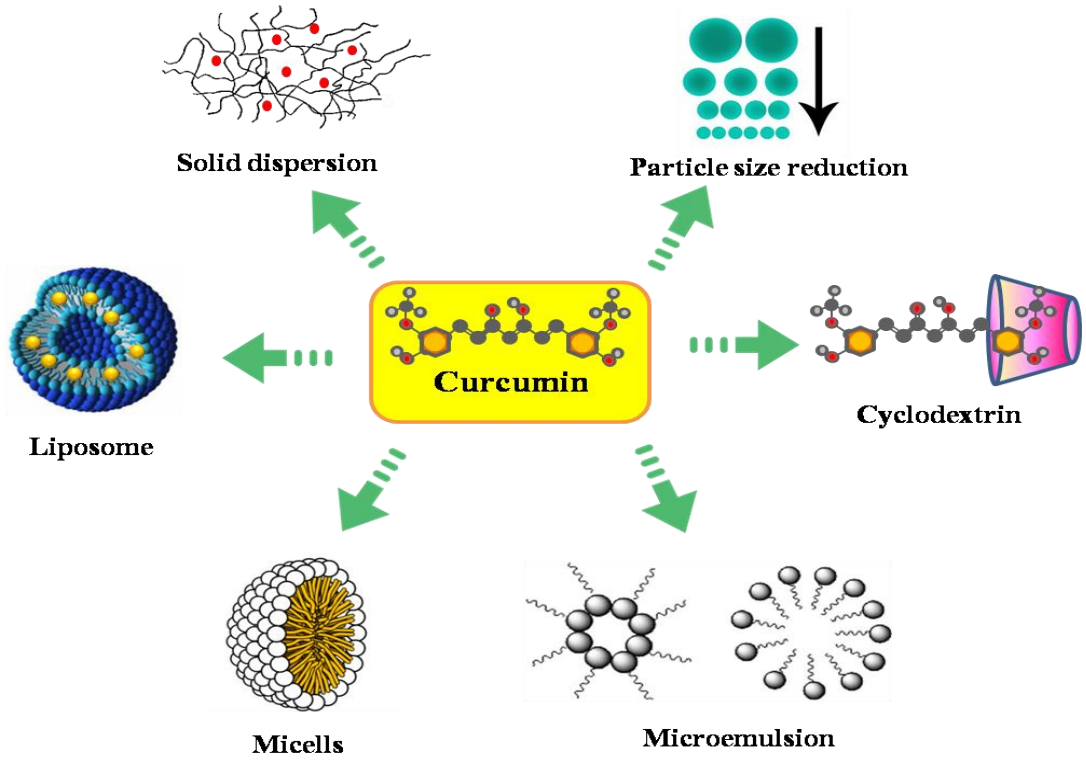


Figure 2-6 Solubility enhancement of curcumin with various techniques adapted from Tiwle et al., 2012.

2.10 Cyclodextrins inclusion complexes

2.10.1 Structure and properties of cyclodextrins

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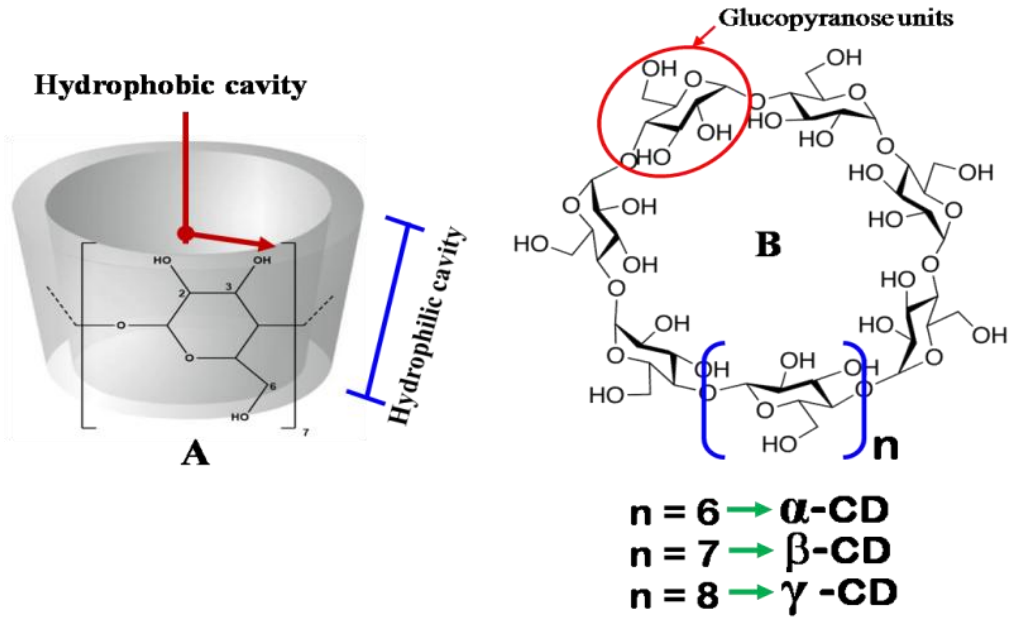

Figure 2-7 Cyclodextrin structure (Brewster & Loftsson, 2007)

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a hydroxyl alkylated CD derivative (Figure 2-8) 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 (Hsu et al., 2013). HP-β-CD is a choice to β-CD having a higher water solubility (above 60%), easy complex formation, low toxic and pharmaceutical and cosmetic products containing HP-β-CD are already on the market (Table 2-9) (Miranda et al., 2011).

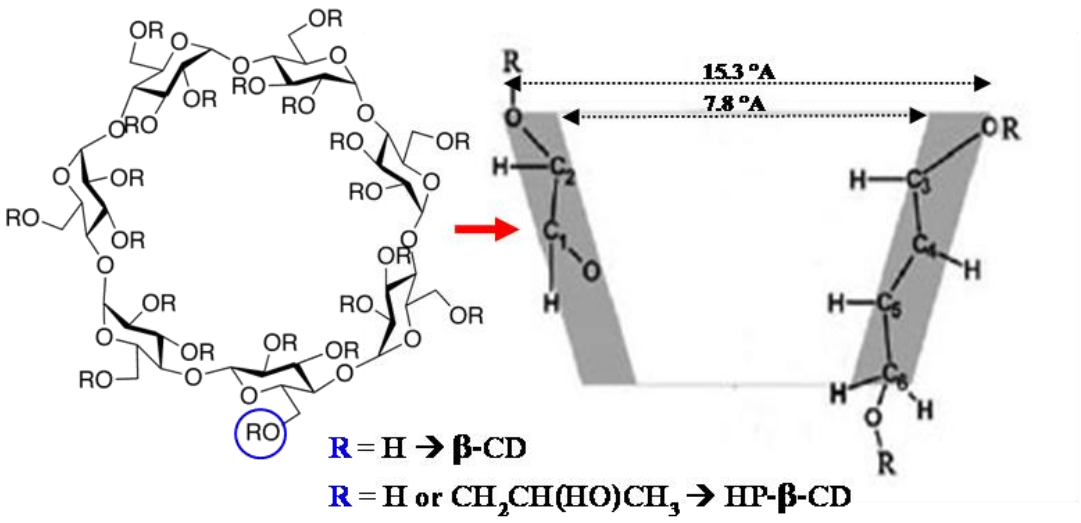

Figure 2-8 β-cyclodextrin and 2-Hydroxypropyl-β-cyclodextrin structures (Loftsson et al., 2005)

Table 2-9 Examples of marketed products containing β-CD and HP-β-CD

Drug	CDs	Administration on route	Trade name	Market
Benexate HCl	β-CD	Oral	Ulgut, Lonmiel	Japan
Dexamethasone	β-CD	Dermal	Glymesason	Japan
Iodine	β-CD	Topical	Nicorette	Japan
Nicotine	β-CD	Sublingual	Nitropen	Japan
Cisapride	HP-β-CD	Rectal	Propulsid	Europe
Hydrocortisone	HP-β-CD	Buccal	Dexocort	Europe
Indomethacin	HP-β-CD	Eye drops	Indocid	Europe
Itraconazole	HP-β-CD	Oral, IV	Sporanox	Europe, USA

2.10.2 Inclusion complex formation

The formation of CDs inclusion complexes, a guest molecule (drug) is inserted into the cavity of the host molecule (CDs) by fitting dimensional between the cavity of host and gest molecule (Brewster & Loftsson, 2007), CDs are able to interact with the large variety of the guest molecules to form non-covalent inclusion complexes. The driving forces for the complex formation are composed of the release of the release of enthalpy-rich water molecules from the cavity, electrostatic interactions, van der Waals interaction, hydrophobic interaction, hydrogen bonding and charge-transfer interactions (Del Valle, 2004). The binding of guest molecules within the CDs is not fixed or permanent but rather a dynamic equilibrium. Binding strength depends on how well the "Host-guest" complex fits together and on specific local interactions between a surface atom (Loftsson et al., 2005). The formation inclusion complex mechanism between drug and CDs were included 4 steps; 1) the water molecules were displaced the polar from the non-polar CDs cavity. 2) Increased number of hydrogen is bonds formed as the displaced water returns to the pool. 3) Reduced the repulsive interactions between the hydrophobic guest molecule and the aqueous environment. 4) Increased hydrophobic interactions as the guest insert itself into the non-polar CDs cavity (Figure 2-9).

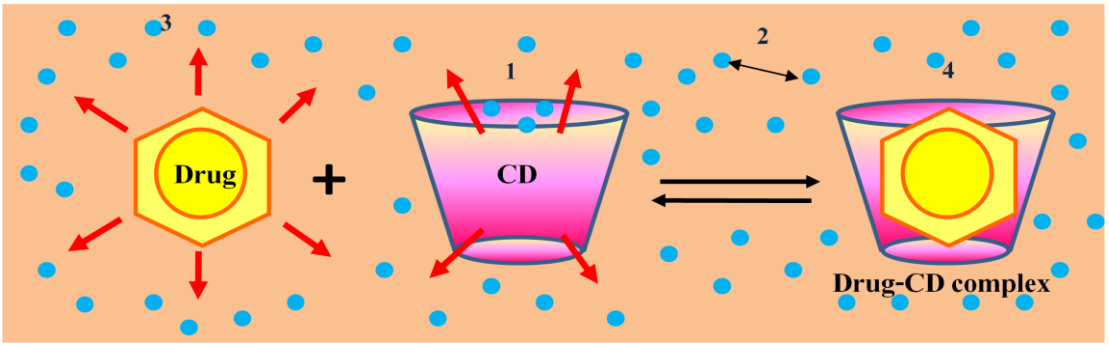

Figure 2-9 The formation drug/CD inclusion complexes (Brewster & Loftsson, 2007)

2.10.3 Phase solubility analysis

Phase solubility is a method to study inclusion complexation, the effect of cyclodextrin (solubilizer) on the substrate (solubilized) which determines not only the value of stability content but also comprehension to the stoichiometry of equilibrium (Higuchi & Conner, 1965). Phase solubility diagram is constructed by plotting between the concentrations of the drug on Y-axis with the concentrations of CDs on the X-axis. Phase solubility diagram are categorized in two major types: A and B profiles (Figure 2-10).

A type profiles indicative of the apparent solubility of the substrate increase as a function of CD concentration. A type has been defined in three subtypes: A_L profile indicate a linear increase in solubility as a function of CDs concentration**,** A_P profile indicates an isotherm wherein the curve deviates in a positive direction from linearity and A_N profile indicate a negative deviation from linearity. **B type profiles** indicated the formation of inclusion complexes with limited water solubility in aqueous poor solubility. B type has been defined in three subtypes: B_S profile indicated complexes of limited solubility, B_I profile indicates insoluble complexes. Generally, the β-CD often gives rise to B type profiles due to their poor water solubility while the chemically modified CDs and their derivatives such as HP-β-CD and SBE-β-CD usually give A type profile because they produce soluble complexes (Brewster & Loftsson, 2007).

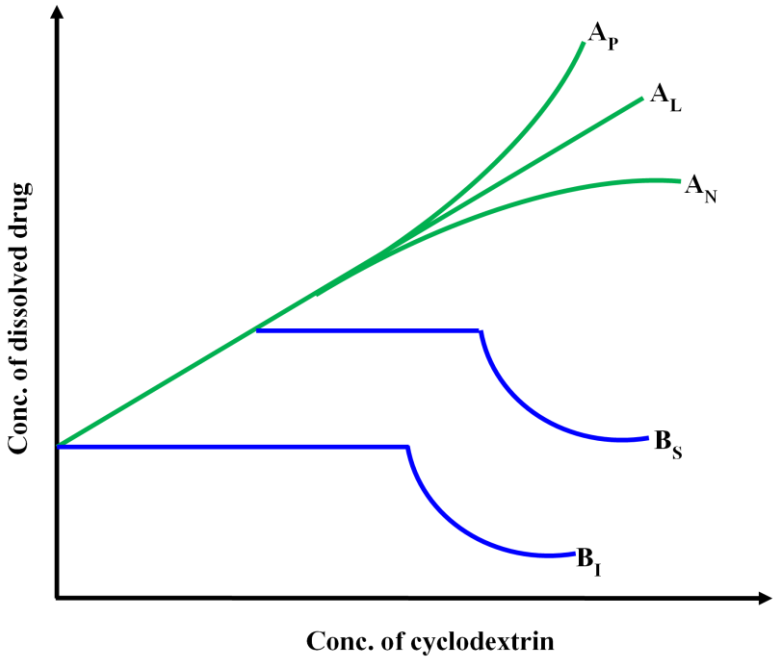

Figure 2-10 Phase solubility diagram (Brewster & Loftsson, 2007)

2.10.4 Pharmaceutical applications of cyclodextrin

There are many applications for CDs in the pharmaceuticals industry. For example, the addition of CDs enhances the water solubility of several poorly water-soluble drugs. Similarity these results in improved bioavailability, enhancing the pharmacological effect approval a reduction in the dose of the drug administered. Inclusion complexes can also promote the handling of volatile products. This can lead to a various way of drug administering, e.g. in the form of tablets. CDs are used to improve the stability of drugs to increase their resistance to oxidation, hydrolysis, light, heat and metal salts. The inclusion of irritating products in CDs can reduces skin damage for the dermal route and also protects the gastric mucosa for the oral route. Moreover, CDs can be applied to reduce the effects of irritant or bitter tasting and bad smelling drugs. It can be summarized in Figure 2-11 (Loftsson & Duchêne, 2007; Tiwari et al., 2010).

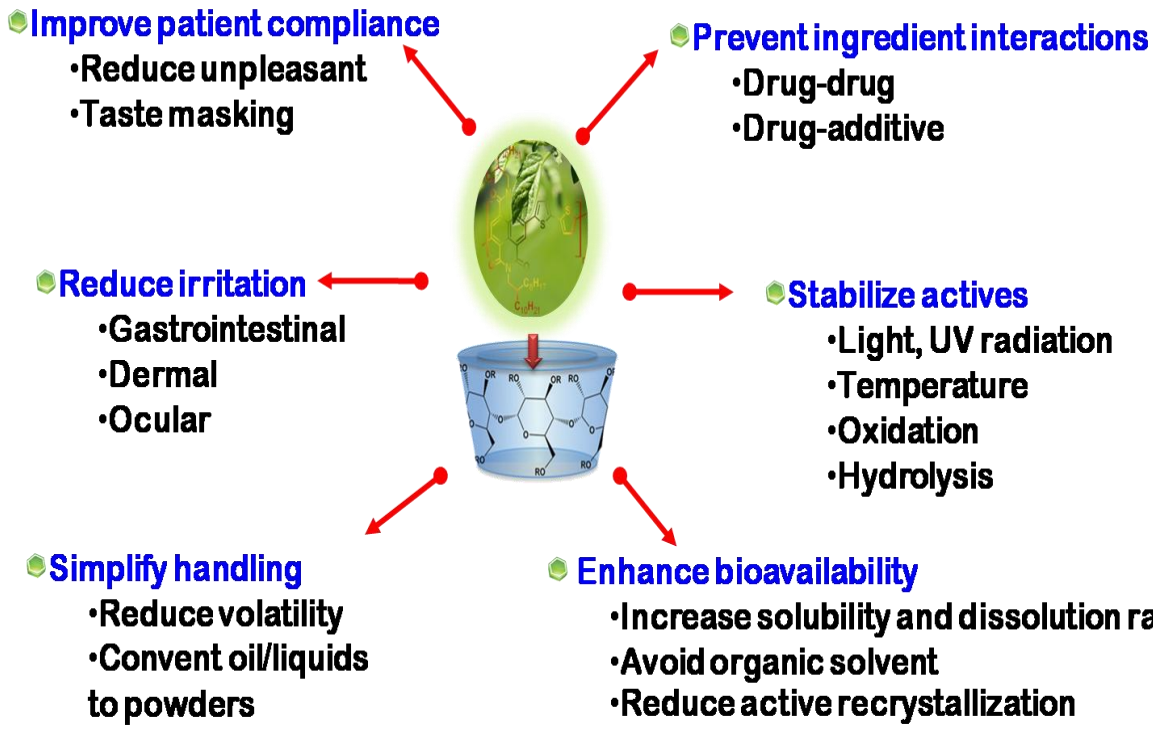

Figure 2-11 Pharmaceutical applications of cyclodextrin (Loftsson et al., 2005)

2.10.5 Cyclodextrin inclusion complex of plant bioactive compounds

Plants are virtually inexhaustible sources of biologically active compounds, which have been widely used in food, cosmetic and pharmaceutical preparations. However, some natural compounds used in dietary supplements and pharmaceutical preparations have limitations due to their low water solubility, stability, or bioavailability. Among the conventional methods used to enhance solubility and bioavailability of drugs, complexing them with CDs seems to be very promising (Pinho et al., 2014; Suvarna et al., 2017).In this review focus on complexes between HP-β-CD and bioactive compounds in herbal plant extract, and present effects of herbal extract CDs complex on the solubility and bioactivity are summarized in Table 2-10, Its application in a pharmaceutical product, dietary supplement products, food and cosmetic industries.

Table 2-10 CDs inclusion complexes of plant bioactive compounds.

Plant/Extracts	Target compound	CDs	Efficacy	References
Isoflavones-rich *Glycine max* extracts (soybean)	daidzein, glycitein and genistein	HP-β-CD	Solubility enhancement	Yatsu et al., 2013
	Daidzein	HP-β-CD, SBE-β-CD	Solubility enhancement	Deng et al., 2016
Rheum rhabarbarum extract (rhubarb)	Anthraquinone (emodin, chrysophanol, rhein, aloeemodin, and physicione)	HP-β-CD	Solubility, cellular uptake and cytotoxicity enhancement	Hsu et al., 2013
Angelica sinensis extract	Ferulic acid, ligustilide , and butylidenephthalide	HP-β-CD	Solubility, cellular uptake and cytotoxicity enhancement	Hsu et al., 2014
Glycyrrhiza glabra L. (licorice)	Glabridin	HP-β-CD	Solubility, antioxidation and antityrosinase inhibitory enhancement	Wei et al., 2017
Tea, berries, fruits, vegetables	Myricetin	HP-β-CD	Solubility and antioxidation enhancement	Yao et al., 2014
Tephrosia barbigerain	Barbigerone	HP-β-CD	Solubility and anticancer enhancement	Qiu et al., 2014
Usnea barbata L.	Usnic acid	β-CD , HP-β-CD	Solubility and antimicrobial enhancement	Nikolic´ et al., 2013

Table 2-10 CDs inclusion complexes of plant bioactive compounds. (continued)

Plant/Extracts	Target compound	CDs	Efficacy	References
Angelica sinensis, *Cimicifuga heracleifolia* and *Lignstcum chuangxiong*	*trans*-Ferulic acid	HP-β-CD	Photostability and solubility enhancement	Wang et al., 2011
Lavandula viridis, *Lavandula pedunculata* subsp. *lusitanica* and *Thymus lotocephalus*	Essential oils	β-CD, HP-β-CD	Antioxidant and solubility enhancement	Costa et al., 2015
Flavonoid-rich *Hypericum perforatum* extracts (St John's wort)	Epicatechin, catechin, quercetin	β-CD	Solubility and antioxidant enhancement	Kalogeropoulos et al., 2010

2.10.6 Cyclodextrin inclusion complex of curcumin

Curcumin has been ravishing major attention because of their broad biological properties with specificity in their action in human health care as pharmaceuticals, nutraceuticals and functional foods (Suvarna et al., 2017). Nevertheless low bioavailability and reduced bioactivity attributed to low solubility and instability is the main drawback hampering the association of these therapeutically potential molecules in drug delivery systems (Liu et al., 2016). Based on the founding of reported study consideration; complexation of curcumin with CDs has occurred to be a promising approach to improve their water solubility, dissolution rate, bioactivity, stability and bioavailability. The present review concludes the complexation of curcumin with cyclodextrin and their derivatives (Table 2-11).

Table 2-11 Complexation of curcumin with cyclodextrins and its derivatives

Compounds	CDs	Methods	Solubility	Effects	References
Curcumin	HP-β-CD	Common solvent evaporation method	Increased solubility 1.36 up to 375.94 μg/mL	Dissolution rate enhancement	Jantarat et al., 2014
Curcumin	HP-β-CD	Kneading method	Increased solubility 0.003 up to 0.0686 mg/mL	Dissolution rate enhancement	Yadav et al., 2009
Curcumin	β-CD	Co-precipitation method	Increased solubility up to 31-fold	Stability enhancement	Mangolim et al., 2014
Curcumin	α-CD, β-CD, γ-CD, DM-β-CD	Freeze-dried method	Increased solubility up to 60, 55, 56 and 1500 folds for α -CD, β-CD, γ-CD, DM-β-CD	Improved solubility and stability	Ansari et al., 2014
Curcumin C3 Complex)	HP-β-CD SBE-β-CD	Autoclave method	Increased solubility up to 15.6 and 13.5 μg/mL	Dissolution rate enhancement	Al - Hagbani & Nazzal, 2017
Curcumin	SBE-β-CD	Lyophilization method	Increased solubility 0.56 up to 102.78 g/mL	Improved antioxidant anticancer activity	Cutrignelli et al., 2014

Table 2-11 Complexation of curcumin with cyclodextrins and its derivatives (continued)

Compounds	CDs	Methods	Solubility	Effects	References
Curcumin 95% purity	β-CD	Co-precipitation method	-	Improved uptake in cancer cell	Yallapu et al., 2010
Curcumin 95% purity	β-CD	Co-precipitation method	-	Improved curcumin delivery and therapeutic efficacy in lung cancer.	Zhang et al., 2016
Curcumin	β-CD	Kneaded and freeze dried methods	-	Improved solubility, dissolusion rate and stability	Darandale et al., 2013
Curcumin 95% purity	β-CD	Co-precipitation method	-	Improved curcumin delivery and therapeutic efficacy in lung cancer.	Zhang et al., 2016
Curcumin	HP-β-CD	solvent evaporation method	-	Improved dissolution rate	Radjaram et al., 2013

2.10.7 Formulation of ternary inclusion complexes

A water-soluble polymer, cyclodextrin (CD) and drug are mixed in a solution to get the so-called ternary inclusion complexes (Figure 2-12); it is possible to enhance drug solubilization, when paralleled to the polymer and CD separately, which is a result of the synergistic effect between these components (Loftsson et al., 1994). An example is a synergistic effect resulting from the addition of HPMC to the complex formed by SBE-β-CD and carbamazepine, with a

consequent enhance in drug solubility in the resulting ternary inclusion complex (Smith et al., 2005). Formulations containing drug: CD complexes with the addition of a polymer have proven to be potent of enhancing the bioavailability of formulations while decreasing the amount of CD by up to 80% (Mura et al., 2001). In the present of water, the polymer aids in the wettability of particles, resulting in an expenited dissolution and enhanced amount of drug delivered *in vitro* (Lahiani-skiba et al., 2006).

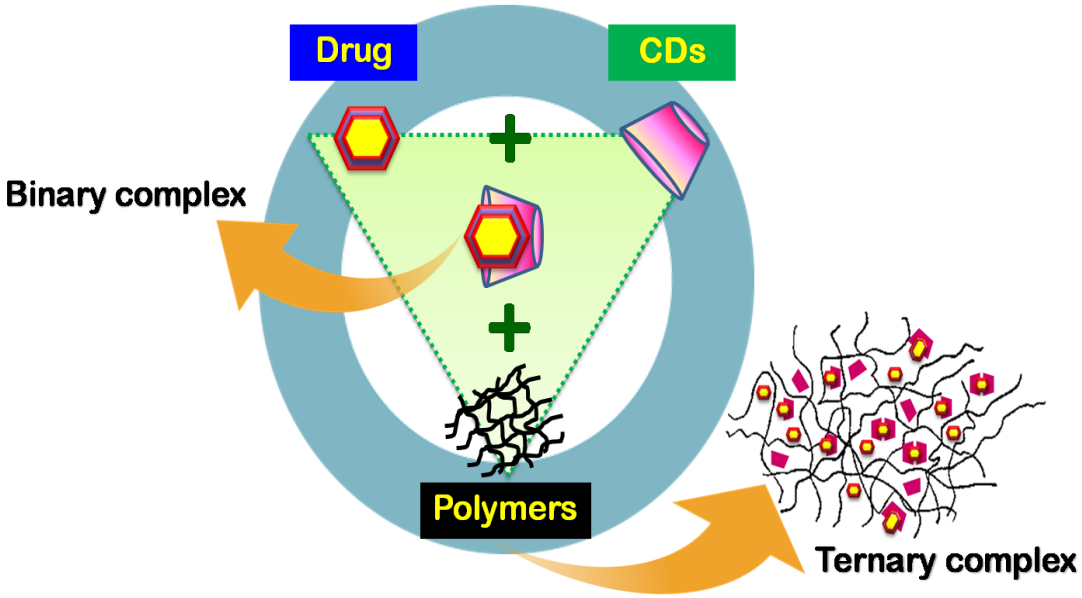

Figure 2-12 Binary and ternary inclusion complex structures were adapted from Savolainen et al., (1998); Loffson et al., (2002); Yadav et al., (2012).

2.10.8 Pharmaceutical applications of ternary inclusion complexes

Most drugs with low solubility have organic solvents, extreme pH conditions in their formulations and emulsifiers, which can cause adverse effects and irritation (Del Valle, 2004). The ternary inclusion complexes can be administered in any dosage form for the treatment of a type of ailments, depending on the biological activity of the complexed drug. Study on ternary inclusion complexes has obtained prominence in recent decades, and it is, Therefor, possible to find a many number of researches in which ternary inclusion complexes obtained for several drugs are summarized (Table 2-12).

Table 2-12 Ternary complexes between drugs, CD and water-soluble polymers

Drug	CD	Water-soluble polymer	Reference
Acetazolamide	HP-β-CD	HPMC, CMC, PVP	Loftsson et al., 2005
Alprazolam	β-CD	HPMC, CMC, PVP	Loftsson et al., 1998
Carbamazepine	SBE-β-CD	HPMC, PVP	Smith et al., 2005
	HP-β-CD	HPMC, CMC, PVP	Brewster and Loftsson, 2007
Celecoxib	HP-β-CD	HPMC, PEG, PVP	Chowdary and Srinivas, 2006
Efavirenz	M-β-CD	PVP	Vieira et al., 2015
Diosmin	β-CD	HPMC, PEG 6000	Anwer et al., 2014
Finasteride	RM-β-CD	HPMC, CMC, PVP	Brewster and Loftsson, 2007
Glimepiride	β-CD, HP-β-CD, SBE-β-CD	HPMC, PEG, PVP	Ammar et al., 2006
Hydrocortisone	HP-β-CD, RM-β-CD	HPMC, CMC, PVP	Loftsson et al., 2005
Lamivudine	β-CD	PVA	Selvam and Geetha, 2008
Meloxicam	HP-β-CD	PVP	El-Maradny et al., 2008
Midazolam	SBE-β-CD	HPMC	Loftsson et al., 2001
Methazolamide	β-CD	HPMC, CMC, PVP	Loftsson and Fridrilksdóttir, 1998
	HP-β-CD	HPMC, CMC, PVP	Loftsson et al., 2005

CMC = carboxymethyl cellulose, HP-β-CD = hydroxypropyl-β-CD, HPMC = hydroxypropyl methylcellulose, PEG = polyethylene glycol, PVP = povidone, RM-β-CD = randomly methylated-β-CD, SBE-β-CD = sulfobutylether-β-CD, EPI-β-CD = Epichlorohydrin-β-CD, M-β-CD = Methyl-β-CD.

2.11 Characterization of CDs inclusion complexes

The involving is up to the mainly on the measurement of the CDs and the component sterical arrangement of the utility associations of the molecules, which leads to a relatively hydrophilic outside and a hydrophobic inside cavity of the molecule. The inclusion complexes formation between the guest and CDs molecules can be evaluated both in solids and solution state in various techniques such as differential scanning calorimetry (DSC), Powder X-ray diffractometry (PXRD), Fourier transforms infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) (Mura, 2015).

2.11.1 Differential scanning calorimetry (DSC): To decide if the guest matter bears to any movements before the thermal degradation of CDs. The shift of the guest matter can be melted. These are also leading to the cause of decomposition, evaporation, oxidation or any way to polymorphic transition. The change of the guest substance indicates the complex formation. The bring about of CDs on the thermo-gram gained by DSC. It was observation for widen, moving and aspect of new pinnacle or fading of assertive pinnacles. Movements in the weight loss were evaluated to provide supporting evidence for the formation of inclusion complexes (Wei et al., 2017). The character of the drug and CDs use and ways of arrangement of compound found that it is consequence the above results appreciably. If the interaction between the drug and the excipient is weak, the shift in the endothermic peak is very small (Anwer et al., 2014).

2.11.2 Powder X-ray diffractometry (PXRD): may be used to detect inclusion complexation in the solid state. When the guest molecules are liquid since liquid has no diffraction pattern of their own, then the diffraction pattern of a newly formed substance clearly differs from that of uncomplexed CDs. Its deviation of dispersion practice points out the compound establishment. Since the guest complex is a solid matter, a collating has to be made in between the diffract-gram of the supposed compound and that of the heterogeneous mixture of particles of the guest and CDs molecules (Yao et al., 2014; Vieira et al., 2015).

2.11.3 Fourier transforms infrared spectroscopy (FTIR): is used to estimate the interaction between CDs and the guest molecules in the solid state. CDs bands often change only slightly upon complex formation and if the fraction of the guest molecules encapsulated in the

complex is less than 25%, bands which could be assigned to the included part of the guest molecules are easily masked by the bands of the spectrum of CDs (Mohan et al., 2012; Yao et al., 2014).

2.11.4 Scanning electron microscopy (SEM): is a microscope which uses electron beam instead of light to illuminate specimen to produce magnified images. Formation of the inclusion complexes can be confirmed by observing the difference in the crystalline state of guest molecules and cyclodextrin under an electron microscope. However, this method is insufficient to assure inclusion complex formation (Lokamatha et al., 2010).

2.12 Toxicology profile of CDs and HP-β-CD

Safety of CDs and derivative were found in many food products and are considered as natural products (Japan), novel food/food additive (EU) and, generally regarded as safe (GRAS) for use as additives/carriers/flavor protectants in food products (US) (Astray et al., 2009). Some of the CDs (γ-CD, HP-β-CD and SBE-β-CD) are also listed as inactive ingredients in approved drug products (USFDA). The oral bioavailability of CDs is very low in animals and humans (0.1 – 3%), CDs are generally not well absorbed intact in the gastrointestinal tract, although renal effects subsequent to systemic absorption are reported (Martina et al., 2013). In the gastrointestinal tract, CDs are hydrolyzed to varying extents depending on the type. The parent CDs are practically resistant to salivary and pancreatic amylases, and all are degraded by colon microflora (Szente & Szejtli, 2004). Chemically modified CDs are more resistant to degradation and are generally excreted intact in feces (Hanumegowda et al., 2014). The LD_{50} or NOEL/NOAELs of CDs and derivative were reported from International Programme on Chemical Safety are summarized in Table 2-13.

HP-β-CD is an alternative to α-CD, β-CD and γ-CD, with improved water solubility properties and may be slightly more toxicological benign. Therefore toxicological profile is an important factor in determined the safety of used for this substance. There are many reported that *in vivo* oral toxicity studies of HP-β-CD were summarized in Table 2-14.

2.12.1 Carcinogenicity studies of HP-β-CD

There are reports in the literature of the findings from an 18-month swiss mouse and a 2-year Wistar rat carcinogenicity study, which both dosed HP-β-CD in the diet, at dose levels of 500, 2000 and 5000 mg/kg body weight/day. In this study, there was no effect on survival and no increase in total tumor incidence of individual tumor type and thus; the study concluded there was no evidence of primary carcinogenic potential in the mouse (Gould & Scott, 2005).

Table 2-13 LD_{50} and NOEL/NOELs of CDs and HP-β-CD

Cyclodextrins		Safety dose
α-CD	LD50	Rat, IV: 1,000 mg/kg
β-CD	LD50	Rat, oral: >5,000 mg/kg
		Rat, IV: 788 mg/kg
		Dog, oral: >5,000 mg/kg
γ-CD	LD50	Rat, IV: >3,750 mg/kg
		Rat, oral: >8,000 mg/kg
HP-β-CD	NOEL/NOAEL	1 year Rat, oral: 500 mg/kg/day
		1mount Dog, oral: 2250 mg/kg/day

LD_{50}: a dose of a chemical which kills 50% of a sample population NOEL: no observed effect level

2.12.2 Human toxicity profile of HP-β-CD

A number of clinical studies are reported in the literature and have shown that HP-β-CD was well tolerated and safe in the majority of patients receiving HP-β-CD at daily oral doses of 4-8 g for at least 2 weeks. After 2 week, the result showed that the volunteers receive higher oral daily doses of 16-24 g leading to increase incidences of soft stools and diarrhea (Irie & Uekama, 1997). Other reports found that intravenous single doses up to 3 g study show no effect on kidney function and well- tolerated by all subject (Seiller et al., 1990). After 1 week of a single dose of 1 g intravenous study, the result showed no adverse effect (Janssen Technical Bulletin, 1992). Therefore, based on these clinical data, HP-β-CD was considered to be non-toxic (at least for 14 days) if the daily dose is <16 g.

Therefore, from previously reported orally administered cyclodextrins at high doses (> 1000 mg/kg/day) may cause reversible diarrhea and cecal enlargement in animals. These effects represent physiologically adaptive responses to a large load of poorly digestible carbohydrates and other osmotically active nutrients, of which the relevance to humans is minimal. All parent cyclodextrins are accepted as food additives and "generally recognized as safe" (GRAS). As a dietary supplement, the total daily oral dose of α-CD may reach 6000 mg/day, for β-CD 500 mg/day and for γ-CD 10000 mg/day, and for HP-β-CD as oral pharmaceutical 8000 mg/day (Cravotto et al., 2006).

Table 2-14 *In vivo* oral toxicity studies of HP-β-CD (Gould & Scott, 2005)

Study duration (days)	Species	Animal nos.	Dose (%) mg/kg/day	Effect
7	Rat	10	4500 (45%)	Increases in ALT, AST, GLDH
7	Rat	10	450 (45%) 2250 (45%) 4500 (45%)	NOEL Loose faeces, clinical pathology (increases in AST, ALT) Loose faeces, clinical pathology (increases in AST, ALT)
28	Rat	20	450 (45%) 4500 (45%)	Increase ALT Increase in water consumption, Loose faeces, increases in lymphocytes, reduction in reticulocyte, HCT, increase in platelet count, increases in ALP, ALT, AST, reductions in creatinine, triglycerides, reduction in glucose concentration
90	Mice and rats	Unknown	500 (Low dose) 1000 (High dose)	Produced transaminase (aspartate and alanine aminotransferase) levels

Table 2-14 *In vivo* oral toxicity studies of HP-β-CD (Gould & Scott, 2005) (continued)

Study duration (days)	Species	Animal nos.	Dose (%) mg/kg/day	Effect
90	dogs and monkeys	Unknown	500 (Low dose) 1000 (High dose)	fecal changes (loose and soft stool)
1 year	Rat	100	500 2000	NOEL Small reduction in body weight, minor haematology and clinical chemistry changes (including increased plasma liver enzymes) and histology changes urinary tract, liver, pancreas
1 year	Dog	Unknown	1000	NOEL
14	Dog	6	540 (45%)	NOEL. No toxicological effects
28	Dog	6	2250 (45%)	NOEL. No toxicological effects

OEL: no observed effect level; ALT: aminotransferase; ALP: amino phosphatase; AST: aspartame transferase; MTD: maximum tolerated dose; GLDH: glutamate dehydrogenas

CHAPTER 3

MATERIAL AND METHODS

3.1 Plant material

The dried powders of *C. longa* rhizomes were obtained from Bangkok Lab & Cosmetic Co., Ltd, Ratchaburi province, Thailand, in October 2015.

3.2 Chemicals and reagents

Standards curcumin (Cu I), demethoxycurcumin (Cu II) and bisdemethoxycurcumin (Cu III) were obtained from Assoc. Prof. Pharkphoom Panichayupakaranant, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Chemicals and reagents used in this study are shown in Table 3-1.

Table 3-1 Chemicals and reagents used in this study

Chemicals	Company, Country
Acetonitile (HPLC grade, 99.9%)	RCI Lab scan Co., Ltd., Thailand
Chloroform (analytical grade, 99.9%)	RCI Lab scan Co., Ltd., Thailand
Diaion® HP-20	Sigma-Aldrich, Germany
Dimethyl sulfoxide (analytical grade, 99.9%)	Sigma-Aldrich, Germany
Dulbecco's modified Eagle medium	Invitrogen Waltham, MA, USA
Ethanol* (commercial grade)	RCI Lab scan Co., Ltd., Thailand
Fetal bovine serum	Gibco, BRL, USA
Formic acid (analytical grade, 98%)	Merck, Germany
Glycerin (BP grade)	Vidyasom Co., Ltd., Thailand
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	Sigma-Aldrich, Germany
Hydrochloric acid (analytical grade, 37%)	RCI Lab scan Co., Ltd., Thailand
Isopropyl myristate (analytical grade, 98%)	Sigma-Aldrich, Germany
Methanol (analytical grade, 99.9%)	Lab scan Asia Ltd., Thailand

Table 3-1 Chemicals and reagents used in this study (continued)

Chemicals	Company, Country
Mineral oil (BP grade)	Vidyasom Co., Ltd., Thailand
Polyethylene glycol 400 (BP grade)	Vidyasom Co., Ltd., Thailand
Polyvinyl pyrrolidone K 30 (BP grades)	Sigma-Aldrich, Germany
Propylene glycol (BP grade)	Vidyasom Co., Ltd., Thailand
Potassium dihydrogen phosphate (analytical grade, 98%)	Ajax Finechem Pty Ltd., Australia
Phosphate buffer saline	Invitrogen Waltham, MA, USA
Silica gel 60 (No. 9385, 230-400 mesh)	Merck, Germany
Sodium chloride (analytical grade, 99%)	Ajax Finechem Pty Ltd., Australia
Sodium hydroxide (analytical grade, 98%)	Ajax Finechem Pty Ltd., Australia
Sulforhodamine B sodium salt	Sigma-Aldrich, Germany
Thin layer chromatography (TLC) silica gel 60 F_{254}	Merck, Germany
Trichloroacetic acid	Carlo Erba, Cornaredo MI, Italy
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific, USA
Trypan blue solution (0.4%)	Thermo Fisher Scientific, USA
Water was purified using a Mill-Q system	Millipore, Bedford, MA, USA

*solvent was redistilled before use.

3.3 Equipment and instrumentals

Equipment and instrumental used in this study are shown in Table 3-2.

Table 3-2 Equipment and instruments used in this study

Instrumentation, model	Company, Country
Analytical balance, FX-3000GD	A&D Co., Ltd., Japan
Analytical balance, AL204	Mettler Toledo, USA
Analytical balance, AB135-S	Mettler Toledo, USA
Biomedical freezer, MDF-U334	Panasonic Co., Ltd., Japan

Table 3-2 Equipment and instruments used in this study (continued)

Instrumentation, model	Company, Country
Biosafety cabinet class II, AC2-451 Airstream	ESCO Co., Ltd., USA
Centrifuge, Kubota 5922	Kubota corporation, Japan
CO_2 incubator, Forma 3111	Thermo Fisher Scientific, USA
Differential scanning calorimeter	Perkin Elmer, MA, USA
Electronic pipette controller, Profiller™ 446	Socorex, Switzerland
Fourier transform infrared spectrometer	Perkin Elmer, CBE, UK
Hot air oven, Memmert	Schwubuch, Germany
Hotplate, IKA C-MAG HS 7	Thermo Fisher Scientific, USA
HPLC, Shimadzu 2017	Shimadzu Co., Ltd., Japan
HPLC column, TSK-gel ODS-80 Tm 25 cm×4.6 mm	Tosho Bioscience, Japan
Inverted microscope, CK2	Olympus Co., Ltd., Japan
Microplate reader, SPECTRO star® Nano	BMG Labtech, Germany
Microwave, MS2127CW	LG Electronics Inc., Thailand
pH meter, MNNV39W	Mettler Toledo, USA
Rotary evaporator, Heidolph™ (Hei-VAP™)	Heidolph, Germany
Stability chamber, HPP260	Memmert, Germany
Stirrer, Variomag Telemodol 40S	H+P Variomag, Germany
Scanning electron microscope, JSM-5200	Jeol Ltd., Tokyo, Japan
Ultrasonic bath, H/T/PC	Crest ultrasonic co., Ltd., USA
USP dissolution apparatus type II, VK 7000	Erweka, Germany
Vacuum pump oil, PC3/RZ6	Vacuubrand, Germany
96-Well microplates, Nunc™	Thermo Fisher Scientific, USA
Water bath, WNB45	Memmert, Germany
Water bath shaker, SW22	Julabo, USA
X-ray diffractometer	Philips, AMS, Netherlands

3.4 HPLC analysis of curcuminoids

3.4.1 Standard solutions

Separate stock solutions of the reference standards Cu I, Cu II and Cu III were prepared in methanol. A working solution of the combined standards was subsequently freshly prepared in methanol, and diluted to provide a series of the standard solutions containing 125, 62.5, 31.25 15.62 and 7.81 μg/mL of each compounds. These solutions were subjected to an HPLC analysis, and the calibration curves were constructed for each of the target analyses by plotting peak areas against concentrations. The linear equations of Y = 68224X - 55754 (r^2 = 0.9999), Y = 92480X + 49438 (r^2 = 0.9999) and Y = 115830X - 56084 (r^2 = 0.9999) correspond to Cu I, Cu II and Cu III respectively.

3.4.2 Sample preparation

The samples were accurately weighed about 2 mg, reconstituted in methanol, and the volume was adjusted to 10 mL in a volumetric flask. These sample solutions were filtered through a 0.45 μm membrane filter, and analyzed immediately in order to avoid possible chemical degradation.

3.4.3 HPLC conditions

HPLC analysis was carried out using the method previously described by Innoue et al. (2008). The stationary phase was a TSK-gel ODS-80 Tm column (25 cm × 4.6 mm). The mobile phase was an isocratic of acetonitrile and 0.1% formic acid in water (50/50 v/v) with a flow rate of 1.50 mL/min. The injection volume was 20 μL, and the detection wavelength was set at 405 nm. The analysis was carried out at 25 °C.

3.5 Extraction of curcuminoids

Extraction of curcuminoids was performed using a microwave-assisted extraction (MAE). The extraction conditions were determined to maximize curcuminoid concentration of the extract. The experiments were as follows.

3.5.1 Determination of suitable solvent

MAE was carried out in a household microwave apparatus, with a microwave frequency of 2450 MHz, and a microwave power of 180 W, at atmospheric pressure. Various solvents, including ethanol, propylene glycol, polyethylene glycol 400 and glycerin (20 mL) were added into a 125 ml-Erlenmeyer flask containing dried powders of *C. longa* (2.0 g) and mixed well and then placed in the microwave irradiation cavity. The extraction process was performed under microwave irradiation for 30 sec. The extracts were then filtered, and subjected to the quantitative HPLC analysis for curcuminoids content. Each experiment was performed in triplicate. The solvents that gave the highest content of curcuminoids were used for the further experiment.

3.5.2. Determination of a suitable powder to solvent ratio

The dried powders of *C. longa* (1, 1.5, 2.0, 1.5 g) were separately extracted with ethanol (20 mL) using MAE at a microwave power of 180 W. The extraction process was performed under microwave irradiation for 30 sec. The extracts were then filtered, and subjected to the quantitative HPLC analysis for curcuminoids content. Each experiment was performed in triplicate. The powder to solvent ratio that gave the highest content of curcuminoids were used for the further experiment.

3.5.3 Determination of a suitable microwave power

The dried powders of *C. longa* (2.0 g) were extracted with ethanol (20 mL) using various microwave powers (180, 360, 600 W). The extraction process was performed under microwave irradiation for 30 sec. The extracts were then filtered, and subjected to the quantitative HPLC analysis for curcuminoids content. Each experiment was performed in triplicate. The microwave power that gave the highest content of curcuminoids was used for the further experiment.

3.5.4 Determination of a suitable microwave irradiation cycles

The dried powders of *C. longa* (2.0 g) were extracted with ethanol (20 mL) using MAE at a microwave power of 180 W, and varied microwave irradiation cycles for 1, 2, 3 and 4 cycles (1 cycle: 30 sec power-on and 30 sec power-off). The extracts were then filtered, and subjected to the quantitative HPLC analysis for curcuminoids content. Each experiment was performed in

triplicate. The microwave irradiation cycles that gave the highest content of curcuminoids was used for the further experiment.

3.5.5 Determination of consecutive extraction times

The dried powders of *C. longa* (2.0 g) were extracted with ethanol (20 mL) using MAE at a microwave power of 180 W for 30 sec. The extraction process was consecutively performed 3 times using the marc and fresh solvent. The obtained extracts were then subjected to the quantitative HPLC analysis for curcuminoids content. Each experiment was performed in triplicate.

3.5.6 Scale-up for preparation of curcuminoid extract

The dried powders of *C. longa* (240 g) were extracted with ethanol (2.4 L) using MAE at 900 W. The extraction process was performed under microwave irradiation for three irradiation cycle (1 cycle: 3 min power-on and 30 sec power-off). The extracts were then filtered, and subjected to the quantitative HPLC analysis for curcuminoids content. The experiment was performed in triplicate. The obtained extract was used for a further study on preparation of curcuminoid-rich *C. longa* extracts (CRE).

3.6 Preparation of CRE

The curcuminoid extract was fractionated by macroporous resins (Diaion® HP-20) to obtain the extracts enriched in curcuminoids. The Diaion® HP-20 (1 kg) was treated with ethanol and loaded into a column (8×100 cm). The column was washed twice with ethanol and equilibrated with 55% v/v ethanol before use. The curcuminoid extract (1.5 L) was dissolved in water (1.2 L). Then the solution was loaded into the Diaion® HP-20 column, and then eluted with 55% v/v, 60% v/v ethanol, respectively. The fractions that contained curcuminoids were pooled, and dried under reduced pressure at 45 °C to produce CRE. The CRE was then subjected to the quantitative HPLC analysis for curcuminoids content. The experiments were performed in triplicate.

3.7 Phase solubility studies

Phase-solubility studies were performed according to the method described by Higuchi and Connors (1965). Briefly, an excess amount of CRE (10 mg) was added to 10 mL of aqueous solution containing various concentrations of HP-β-CD (0-60 mM). The content was shaken in shaking water bath at 25 ± 1 ºC. After achieving equilibrium (48 h) the solution was filtered through a 0.45 µm membrane filter paper. The sample was diluted suitably and assayed for curcuminoid content by HPLC. Each experiment was carried out in triplicate. The phase-solubility diagram was obtained by plotting between curcuminoid solubility and HP-β-CD concentrations. The apparent stability constants (K_s) were calculated from the slope and intercept of line of phase solubility diagram with the assumption of 1:1 stoichiometry, according to the following equations.

$$\mathrm{Ks} = \frac{\mathrm{Slope}}{\mathrm{Intercept}\,(1 - \mathrm{Slope})}$$

Ks = stability constant

Intercept = the intrinsic solubility of curcuminoids in absence of HP-β-CD

3.8 Preparation of CRE-cyclodextrin inclusion complex

The preparations of CRE were followed by the section 3.6. Finally procedure, the obtain CRE solution was directly used for study in the section 3.8.1 and 3.8.2 without solvent evaporation.

3.8.1 Determination of molar ratios for preparation of CRE:HP-β-CD binary inclusion complex

The binary inclusion complex was prepared by a solvent evaporation method (Ghosh et al., 2011). Various molar ratios of CRE and HP-β-CD (1:0.5, 1:1, 1:1.5, 1:2) were used for the preparation of the CRE:HP-β-CD binary inclusion complex. HP-β-CD was added into the CRE solution (1.5 L), and stirred for 24 h, at a room temperature. The reaction mixtures were then dried under reduced pressure at 45°C, and then dried in a hot air oven at 45°C for 24 h. The dried complexes were powdered, and passed through a No. 45 sieve. The binary inclusion complexes

were kept in a well-closed container protected from light, and stored in a desiccator, at room temperature. These binary inclusion complexes were subjected to determination of curcuminoids solubility and curcuminoids entrapment (CE). The molar ratio that gave the highest solubility of curcuminoids and CE was used for further studies.

3.8.2 Determination of PVP K30 concentration for preparation of CRE:HP-β-CD:PVP K30 ternary inclusion complex

The ternary inclusion complexes were prepared by a solvent evaporation method. HP-β-CD was added into the CRE solution (1.5 L) at 1:1 molar ratio, and stirred for 24 h, at a room temperature. Various concentration of PVP K30 (5, 7, 9, 11% w/w of the solid binary complex) was then added into the reaction mixture, and stirred for 1 h, at room temperature. The reaction mixtures were then dried under reduced pressure at 45°C, and then dried in a hot air oven at 45°C for 24 h. The dried complexes were powdered and passed through a No. 45 sieve. The ternary inclusion complexes were kept in a well-closed container protected from light, and stored in a desiccator at room temperature. These ternary inclusion complexes were subjected to determination of curcuminoids solubility and CE. The concentration of PVP K30 that gave the highest solubility of curcuminoids and CE was used for further studies.

3.8.3 Preparation of binary and ternary physical mixtures

The binary mixture of CRE and HP-β-CD was prepared in a 1:1 (molar ratio), and a ternary mixture of CRE, HP-β-CD and PVP K30 was prepared using CRE and HP-β-CD (1:1 molar ratio) and PVP K30 (9% of the binary mixture). All components were blended in a mortar for 10 min to obtain a homogenous powder.

3.9 Determination of reaction time for scale up preparation of binary inclusion complex

The preparations of CRE were followed by the section 3.6. Finally procedure, the obtain CRE solution was directly used for experiment study without solvent evaporation.

HP-β-CD was added into the CRE solution (1.5 L) at 1:1 molar ratio, and stirred at room temperature, for 24, 48, and 72 h, respectively. The solutions were dried under reduced pressure at 45°C, and then dried in a hot air oven at 45°C for 24 h. The dried complexes were powdered,

and passed through a No. 45 sieve. The binary inclusion complex was kept in a well-closed container protected from light, and stored in a desiccator at room temperature. These binary inclusion complexes were subjected to determination of solubility of curcuminoids. The reaction time that gave the highest curcuminoids solubility was used for scale-up preparation of binary and ternary inclusion complexes.

3.10 Scale-up production of CRE, binary and ternary inclusion complexes

The scale-up production conditions of CRE, binary and ternary inclusion complexes were obtained optimal conditions derived from the experiments in section 3.6, 3.8 and 3.9. These processes were included 3 steps, including extraction, fractionation, and preparation of the inclusion complexes.

Extraction: The dried powders of *C. longa* (720 g) were extracted with ethanol (7.2 L) using MAE at 900 W, with three irradiation cycles (1 cycle: 3 min power-on, and 30 sec power-off). The extracts were then filtered through a filter paper. The obtained curcuminoid extracts (4.5 L) was directly used in the fractionation process without solvent evaporation.

Fractionation: The curcuminoid extracts was fractionated on four Diaion® HP-20 columns to obtain the extracts enriched in curcuminoids. On each column (3 columns), the ethanol extracts (1.5 L) was dissolved in water (1.2 L), loaded on to the Diaion® HP-20 column, and then eluted with 55% v/v ethanol in water (5 L), and 60% v/v ethanol in water (10 L), respectively. The first obtain fractions (1.3 L) was discarded, and then the later eluted fractions (13 L) that contains curcuminoids was collected. The obtain CRE from each column was mixed (total volume 36 L). The CRE solution was then subjected to the quantitative HPLC analysis for curcuminoids content. It was directly used for preparation of the inclusion complexes without solvent evaporation.

Preparation of the inclusion complexes: For binary inclusion complex, HP-β-CD was added into the CRE solution (13 L) at 1:1 molar ratio, and stirred for 48 h at room temperature. For ternary inclusion complex, HP-β-CD was added into the CRE solution (13 L) at 1:1 molar ratio, and stirred for 48 h at room temperature, and then PVP K30 (9% w/w of the solid complex) was added. The reaction mixture was mixed and stirred for 1 h, at room temperature. CRE, binary and ternary inclusion complexes solution were dried under reduced pressure at 45°C, and then

dried in a hot air oven at 45°C for 24 h. The dried CRE, binary and ternary inclusion complexes were powdered and passed through a No. 45 sieve, and kept in a well-closed container protected from light, and stored in the desiccator at room temperature. This process is summarized in figure 3-5.

The scale-up powder of CRE, binary and ternary inclusion complexes were used for further studies on solubility, characterization of solids complexes, dissolution, stability and *in vitro* anticancer activity.

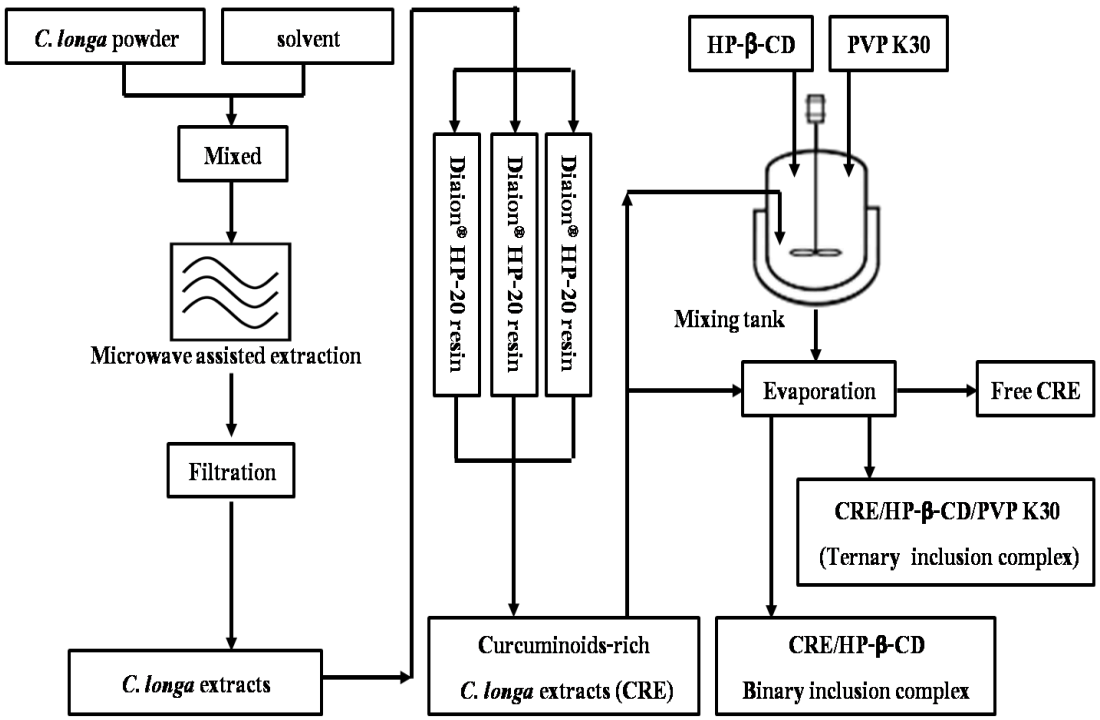

Figure 3-1 Scale-up production of CRE, binary and ternary inclusion complexes

3.11 Solubility study

The solubility of CRE and the binary and ternary inclusion complexes was studied in distilled water at 25 ± 1ºC. The excessive amount of each inclusion complex (~5 mg) and CRE was separately placed in a 20 mL-vial containing 10 mL of distilled water, and shacked for 24 h at 25 ± 1ºC until a saturated solution was obtained. The dispersion sample was then centrifuged at 1,000 rpm and the supernatant was filtered through a 0.45 μm filter. The filtrate was diluted with methanol and the volume adjusted to 10 mL in a volumetric flask. The content of curcuminoid was analyzed using the quantitative HPLC analysis of curcuminoids. The experiment was performed in triplicate.

3.12 Curcuminoid entrapment

Each sample containing 2 mg curcuminoids was accurately weighed and placed in a 20 ml-vial. The samples were extracted with 2 mL ethanol using an ultrasonic sonicator for 30 min. The solution was filtered through a 0.45 μm filter. The filtrate was diluted with methanol and the volume adjusted to 10 mL in a volumetric flask. The content of curcuminoids was analyzed by using the quantitative HPLC analysis of curcuminoids. The experiment was performed in triplicate.

$$\% \text{ CE} = \frac{\text{Actual total curcuminoids concentration}}{\text{Theoretical total curcuminoids concentration}} \times 100$$

3.13 Characterization of the inclusion complexes

3.13.1 Fourier transforms infrared spectroscopy (FTIR)

The infrared spectra of CRE, HP-β-CD, PVP K30, binary and ternary physical mixtures, and binary and ternary inclusion complexes were analyzed using an FTIR spectrometer. Pellets of the sample in KBr were prepared on KBr press. The spectra were scanned over a wave number of 4000 to 500 cm^{-1}.

3.13.2 X-ray diffractometry (PXRD)

The PXRD patterns of CRE, HP-β-CD, PVP K30, binary and ternary physical mixtures, and binary and binary and ternary inclusion complexes were analyzed using an X-ray diffractometer. The samples were irradiated with monochromatized Cu-k $_{\infty}$ radiation and analyzed between 0 to 55 $^{\circ}\theta$. The voltage and current used were 30 kV and 25 mA, respectively.

3.13.3 Differential scanning calorimetry (DSC)

The thermal characteristics of CRE, HP-β-CD, PVP K30, binary and ternary physical mixtures, and binary and ternary inclusion complexes were determined using a differential scanning calorimeter. Sample (~2 mg) was added into an aluminum pan and hermetically sealed. The scanning rate was 10°C/min, and the scanning temperature range of 50 to 400 °C under nitrogen flow of 20 mL/min. The empty pan was used as the reference.

3.13.4 Scanning electron microscopy (SEM)

The morphologies of CRE, HP-β-CD, PVP K30, binary and ternary physical mixtures, and binary and ternary inclusion complexes were analyzed using a scanning electron microscope. Samples were coated with a thin gold-palladium layer using a sputter coater unit, and the surface topography was operated at an acceleration voltage of 10 kV.

3.14 Dissolution study

The dissolution of CRE, CRE binary and ternary inclusion complexes were evaluated using the curcuminoid release profiles. The study was carried out in the USP dissolution apparatus type II using the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) without enzyme as a dissolution media. The CRE, CRE binary and ternary inclusion complexes containing 20 mg curcuminoids were accurately weighed and placed in a vessel containing 200 mL SGF or SIF. The temperature was controlled at 37 ± 0.5ºC, and the paddle was set at 75 rpm throughout the study. At fixed time intervals (3, 6, 10, 15, 30, 45, 60 min) 2 mL aliquots were withdrawn, and equal volumes of fresh medium were replaced. The samples were filtered. The filtrate was diluted with methanol and the volume adjusted to 10 mL in a volumetric flask and assayed for curcuminoid content using the HPLC method. The release profiles of curcuminoids of CRE, CRE

binary and ternary inclusion complexes were established and compared to each other. The experiment was performed in triplicate.

3.15 Stability determination

Stability determinations of CRE as well as the CRE, binary and ternary inclusion complexes were carried out using the methods previously reported by Puttarak et al. (2010).

3.15.1 Effect of temperature on stability

The samples were weighed about 200 mg and stored in a well-closed container, protected from light, and stored at 4 ± 1°C and a control room temperature (25 ± 1°C) for 4 months.

3.15.2 Effect of accelerated condition on stability

The samples were weighed about 200 mg and stored in a well-closed container, protected from light, and stored in the stability chamber at 45 ± 1°C, 75% humidity for 4 months.

An accuracy weight of the sample was taken at 1, 2, 3 and 4 months, and subjected to the quantitative HPLC analysis of curcuminoids. The initial amounts of total curcuminoids in the samples were defined as 100%. The means amounts of curcuminoids for each sampling time were calculated as percentage remains. The samples extract were defined as a stable samples when the percentage of curcuminoid remains was of not less than 90%. The experiment was performed in triplicate.

3.16 *In vitro* anticancer activity evaluation

3.16.1 Cell culture

The human lung adenocarcinoma (A-549), human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma (HT-29) cell lines were from The National Cancer Institute, Bangkok, Thailand. The cancer cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and maintained at 37ºC, 95% relative humidity, and CO_2 of fewer than 5%.

3.16.2 Anticancer activity assay

The *in vitro* anticancer activity of CRE and the inclusion complexes against cancer cell lines were determined using the sulforhodamine B (SRB) assay (Yuenyongsawad et al., 2014). Briefly, cells growing as monolayer in a 25 cm^3 culture flask were washed with phosphate buffer, pH 7.4 and trypsinized with 0.25% trypsin-EDTA to make a single-cell suspension. The viable cells were counted by trypan blue exclusion in a haemacytometer, and dilute with medium to give a final concentration of 4×10^4 cells/mL, and 100 µL/well of these of cell suspension were seeded in a 96-well microplate and incubated at 37°C to allow for cell attachment. After 24 h the cells were treated with 100 mL of sample solutions at various concentrations (25 µg/mL crude extract or a five-fold diluted pure compound in medium) and incubated at 37°C for 72 h. After an incubation period, the cellular proteins were fixed with 100 µL of cold 10% (w/v) TCA to each well, and the plate was incubated at 4°C for at least 1 h. The plate was then washed with water for four washing cycles, dried completely at room temperature, and stained with 50 mL 0.4% SRB solution in 1% acetic acid in each well (allowed to stain for 30 min). The dye was then dissolved in Tris base solution (pH 10.5) and shaken for 5 min. The percentage of cell growth inhibition was determined by measuring the absorbance at 492 nm. The activities were reported as IC_{50} value. The IC_{50} value (effective concentration of sample required to inhibit cell growth by 50%) was calculated from dose-response curves plotting between % inhibition and concentrations, according to the following equations.

$$\% \text{ Inhibition } = \frac{(\text{OD control} - \text{ OD sample})}{\text{OD control}} \times 100$$

3.17 Statistical analysis

The curcuminoids contents were expressed as mean ± S.D. The statistical significance was calculated by analysis of variance (ANOVA), followed by Tukey's test. ($P < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative HPLC analysis of curcuminoids content

In this study, three curcuminoids, curcumin (Cu I), demethoxycurcumin (Cu II) and bisdemethoxycurcumin (Cu III) were used as the standard markers for quantitative HPLC analysis using the method previously described (Innoe et al., 2008). All these curcuminoids were eluted within 20 min with satisfactory resolution (Figure 4-1). The calibration curves of the three curcuminoids were established at the concentrations between 7.81 – 250 μg/mL. The linear equations of Y = 68224 – 55754 (r^2 = 0.9999), Y = 92480X + 49438 (r^2 = 0.9999) and Y = 115830X – 56084 (r^2 = 1.0000) corresponded to Cu I, Cu II and Cu III, respectively.

4.2 Determination of the suitable solvent

It has been reported that acetone and ethyl acetate were the most appropriate solvent for extraction of curcuminoids from *C. longa* powders and mostly used for preparation of curcuminoid extracts in the industrial production (Li et al., 2011). However, they are a dangerous and expensive organic solvent. A search for an alternative green solvent for extraction of curcuminoids from *C. longa* powders is therefore very interested. The present study, some green solvents, i.e. ethanol, propylene glycol (PG), polyethylene glycol 400 (PEG400) and glycerin, were evaluated for their ability to extract curcuminoids from *C. longa* powders. The result showed that ethanol produced the highest content of the curcuminoids and extraction yield (5.58 mg/mL, 60% yield), followed by PG, PEG400, and glycerin, respectively (Table 4-1 and Figure 4-2). This implies that besides the dielectric constant of solvents, viscosity of solvent, and solubility of target compounds also play a major role in MAE. Ethanol was therefore selected as the suitable green solvent for further studies on optimization of MAE conditions (Puttarak & Panichayupakaranant, 2013).

Interestingly, using PG as an alternative green solvent, the obtained extract can be directly used for further drug formulations without solvent evaporation before use. For example, we can

directly use the PG extract of curcuminoids for formulation of curcuminoid creams, curcuminoids in self-microemulsion systems for further oral drug development (Setthacheewakul et al., 2010). Furthermore, we are able to reduce the preparation step in the production line. Therefore, we can save time and cost-effective. This result suggested that ethanol was most effective solvent for extraction of Cu I, Cu II and Cu III which provided curcuminoid content of 4.2, 6.7 and 12.5 mg/mL, respectively. Therefore, we were selected ethanol as one of the best solvent for further studies on optimization of MAE parameter.

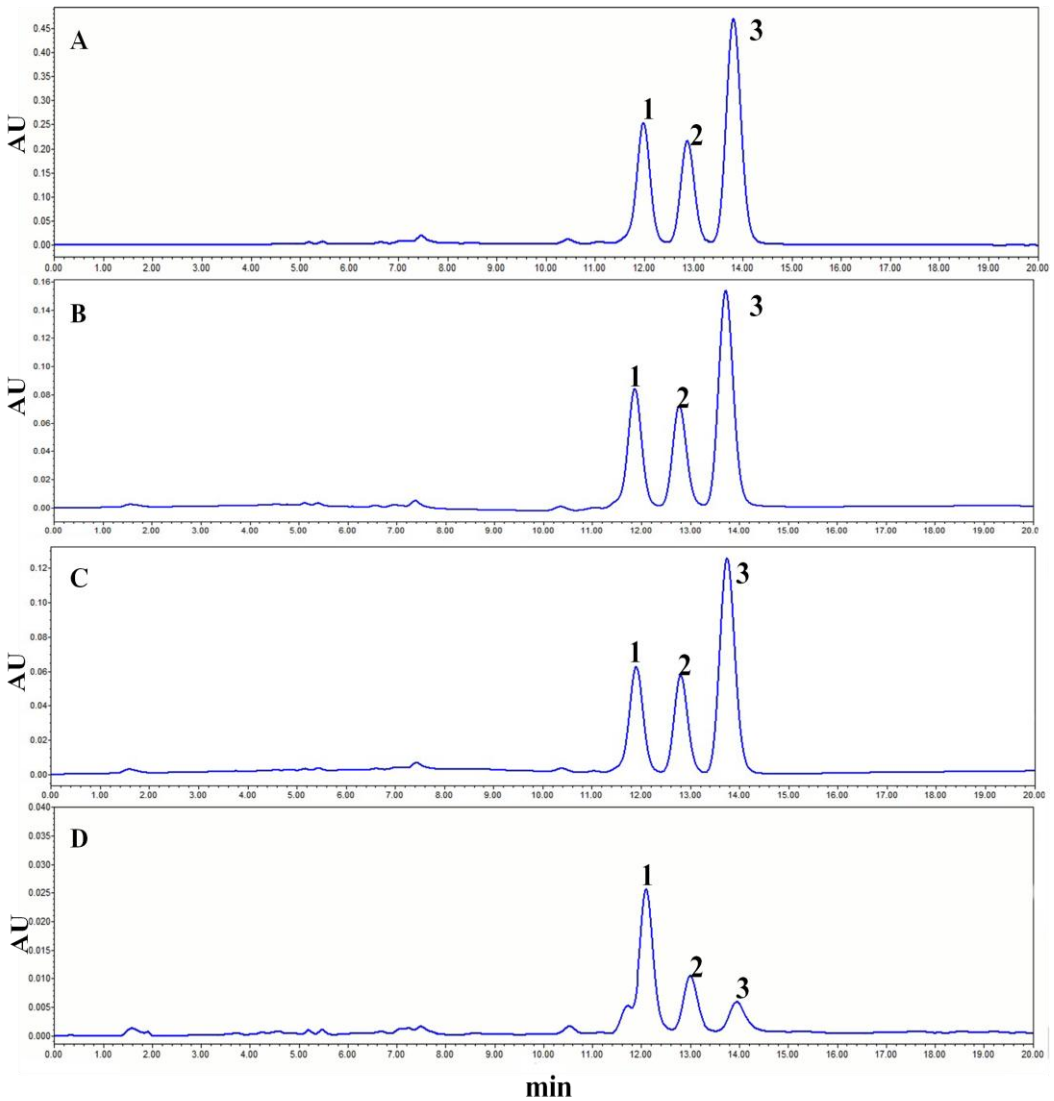

Figure 4-1 HPLC-chromatograms of curcuminoid extracts; Ethanol (**A**), PEG400 (**B**), Propylene glycol (**C**) and Glycerin (**D**) extracts from *C. longa* powders. **1** = Bisdemethoxycurcumin; **2** = Demethoxycurcumin; **3** = Curcumin.

Table 4-1 Curcuminoids content of C. *longa* extracts, extracted under MAE conditions with various solvents

Solvents	Curcuminoids content (mg/mL)				Volume of extract (mL)	Total curcuminoids (mg)
	Cu I	Cu II	Cu III	Total		
Ethanol	3.58 ± 0.19	1.10 ± 0.04	1.01 ± 0.01	5.68 ± 0.24[a]	15	82.41 ± 3.44
PG	2.59 ± 0.19	0.83 ± 0.06	0.77 ± 0.05	4.18 ± 0.29[b]	15	58.56 ± 4.09
PEG400	2.81 ± 0.15	0.66 ± 0.04	0.58 ± 0.04	3.42 ± 0.22[c]	14	46.17 ± 3.17
Glycerin	ND[*]	ND[*]	0.11 ± 0.01	0.11 ± 0.01[d]	9	2.18 ± 0.04

[*]ND: Not Detected

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

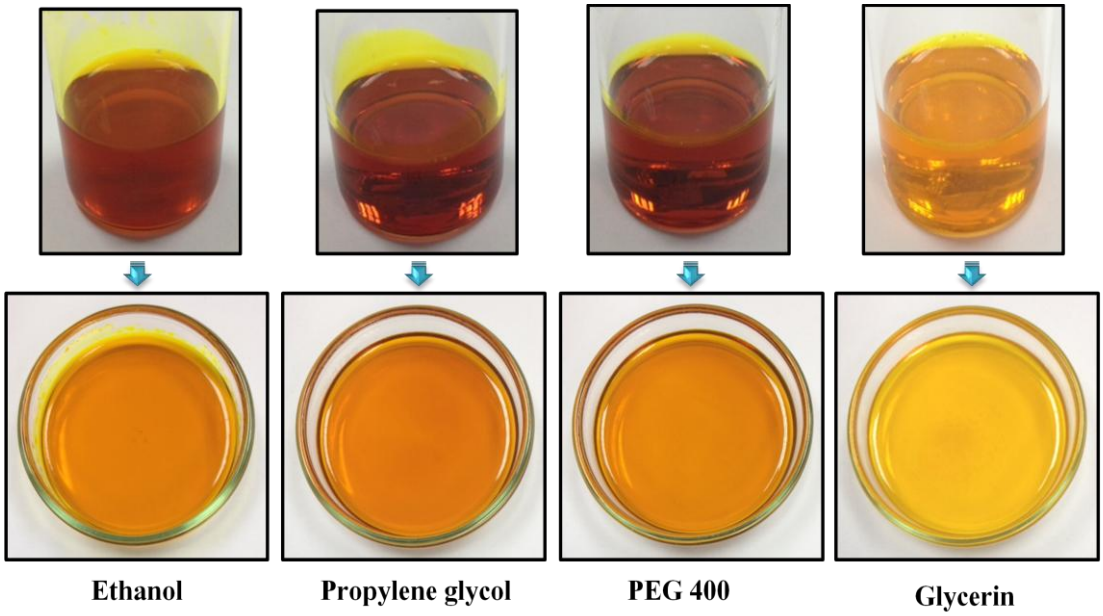

Figure 4-2 Physical appearances of *C. longa* extracts, extracted under MAE conditions with various solvents

4.3 Optimization of MAE conditions

The variable factors for the MAE operating parameters included the powders of *C. longa* to solvent ratios (1:20, 1.5:20, 2.0:20 and 2.5:20 g/mL), microwave irradiation power (180, 360 and 600 W), the numbers of microwave irradiation cycles (1,2,3 and 4 cycles; 1 cycle: 30 sec

power on, and 30 sec power off) and consecutive extraction times (1, 2 and 3 times) were determined by single-factor experiments.

4.3.1 Determination of a suitable powder to solvent ratio

The effect of C. *longa* powders to solvent ratios on curcuminoids extraction were determined by varying the ratios to be 1.0:20, 1.5:20, 2:20, and 2.5:20 (g/mL). The results demonstrated that increasing of *C. longa* powders to solvent ratio resulted in an increase in the curcuminoid content (Table 4-2). However, the curcuminoids content and extraction yield in 2.5:20 and 2.5:20 g/mL were not significantly different. Therefore, 2 g of *C. longa* powder 20 mL of ethanol was used for a further study on the effect of microwave power on the extraction of the curcuminoids.

Table 4-2 Curcuminoids content of *C. longa* extracts, extracted under MAE conditions with various ratios

Ratio (g/20mL)	Curcuminoids content (mg/mL)				Volume of extract (mL)	Total curcuminoids (mg)
	Cu I	Cu II	Cu III	Total		
1.00 g	2.42 ± 0.08	0.71 ± 0.02	0.65 ± 0.02	3.78 ± 0.12[a]	15	58.60 ± 1.93
1.50 g	3.57 ± 0.07	1.03 ± 0.02	0.96 ± 0.02	5.56 ± 0.11[b]	15	86.25 ± 1.72
2.00 g	4.71 ± 0.13	1.38 ± 0.07	1.26 ± 0.07	7.35 ± 0.19[c]	14	106.57 ± 2.77
2.50 g	4.76 ± 0.06	1.44 ± 0.02	1.33 ± 0.04	7.52 ± 0.13[c]	14	109.06 ± 1.84

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.3.2 Determination of a suitable microwave power

The effect of the irradiation power was evaluated. The result showed that increasing the irradiation power up to 600 W did not affect the curcuminoids content in the ethanol extracts of *C. longa* powders (Table 4-3). This suggested that increasing the microwave irradiation powers could not increase an extraction capacity of ethanol for curcuminoids. Therefore, the irradiation power was optimized at 180 W for further studies.

4.3.3 Determination of a suitable microwave irradiation cycles

Table 4-3 Curcuminoids content of C. *longa* extracts, extracted under MAE conditions with various irradiation powers

Irradiation power	Curcuminoids content (mg/mL)				Volume of extract (mL)	Total curcuminoids (mg)
	Cu I	Cu II	Cu III	Total		
180 W	4.43 ± 0.13	1.30 ± 0.03	1.16 ± 0.01	6.89 ± 0.17 [a]	14	99.90 ± 2.48
360 W	4.29 ± 0.32	1.26 ± 0.09	1.14 ± 0.09	6.69 ± 0.50 [a]	14	97.04 ± 7.31
600 W	4.40 ± 0.30	1.33 ± 0.03	1.16 ± 0.10	6.89 ± 0.42 [a]	14	99.85 ± 6.01

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different ($P < 0.05$) when compared to means.

It is necessary to optimize a proper irradiation cycle to complete the extraction process. Different irradiation cycles from 1 to 4 cycles were examined for the extraction of curcuminoids. Increasing the number of irradiation cycles (one cycle is 30 sec power on and 30 sec power off) did not increase the curcuminoids content in the extracts (Table 4-4). Only one cycle was sufficient for extracting curcuminoids by MAE. Therefore, one cycle of irradiation was used for further studies on the effect of the extraction cycles.

Table 4-4 Curcuminoids content of C. *longa* extracts, extracted under MAE conditions with various irradiation cycles

Irradiation cycles	Curcuminoids content (mg/mL)				Volume of extract (mL)	Total curcuminoids (mg)
	Cu I	Cu II	Cu III	Total		
1	4.27 ± 0.06	1.27 ± 0.02	1.14 ± 0.02	6.68 ± 0.09 [a]	14	96.87 ± 1.28
2	4.37 ± 0.03	1.30 ± 0.01	1.17 ± 0.01	6.84 ± 0.04 [a]	14	99.12 ± 0.54
3	4.41 ± 0.09	1.31 ± 0.02	1.19 ± 0.02	6.91 ± 0.13 [a]	14	100.19 ± 1.93
4	4.41 ± 0.18	1.24 ± 0.05	1.14 ± 0.05	6.56 ± 0.28 [a]	14	95.05 ± 1.05

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different ($P < 0.05$) when compared to means.

4.3.4 Determination of consecutive extraction times

The effect of successive extractions of the residue such as the number of cycles extracted was determined. The extracted residue was re-extracted using fresh solvent. After the first extraction, the amount of the additional curcuminoids was very low. Almost 90% of the total curcuminoids was obtained by the first extraction (Table 4-5). It is, therefore, not worthwhile to perform the consecutive extractions using fresh solvent.

The optimal conditions of MAE for small-scale preparation of curcuminoids extract are suggested as follows: use 2 g of dried powders of *C. longa* extracted with 20 mL of ethanol at a microwave power of 180 W, for 30 sec and without consecutive extraction.

Table 4-5 Curcuminoids content of C. *longa* extracts, extracted under MAE conditions with various extraction times

Extraction times	Curcuminoids content (mg/mL)				Volume of extract (mL)	Total curcuminoids (mg)
	Cu I	Cu II	Cu III	Total		
1	4.48 ± 0.21	1.32 ± 0.06	1.17 ± 0.05	6.97 ± 0.32[a]	14	101.08 ± 4.71
2	0.29 ± 0.01	ND	ND	0.29 ± 0.01[b]	15	4.41 ± 0.13
3	ND	ND	ND	ND	15	ND

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different ($P < 0.05$) when compared to means.

4.3.5 Scale-up for preparation of curcuminoid extract

MAE conditions were determined for an extraction of 240 g dried powders of *C. longa* with 2.4 L of ethanol using a 900 W microwave. The extraction conditions were modified based on the small-scale MAE conditions described above. An extraction efficiency of MAE is usually dependent on the temperature of extraction, which relates to the radiation power and time. The present study showed that the extraction conditions needed three irradiation cycles (one cycle is 3 min power on, and 30 sec off) to obtain the curcuminoids extract containing total curcuminoid concentration 7.78 ± 0.74 mg/mL (Table 4-6). This curcuminoids extract was used for further studies on preparation of CRE.

Table 4-6 Curcuminoids content of C. *longa* extracts, extracted under MAE conditions with various scale.

Scale	Curcuminoids content (mg/mL)				Extraction Yield (%)
	Cu I	Cu II	Cu III	Total	
Small	4.49 ± 0.21	1.32 ± 0.06	1.17 ± 0.21	6.98 ± 0.32[a]	72.5
Large	4.93 ± 0.48	1.51 ± 0.13	1.34 ± 0.01	7.78 ± 0.74[a]	71.6

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.4 Preparation of CRE

On the basis of HPLC analysis, the crude ethanol extracts of *C. longa* obtained from MAE method contained total curcuminoids content of 27.60% w/w dry weight extract, in which the amount of Cu I, Cu II, and Cu III were 17.56, 5.34, and 4.70% w/w, respectively (Table 4-7). It has shown the low curcuminoids content and high content of oleoresin, non-volatile compounds and other pigments in extracts (Figure 4-3A). The ethanol extracts of C. *longa* were subjected to purification using Daion® HP20, in order to concentrate the curcuminoids in *C. longa* extracts as well as to decrease the other compounds**.** It was shown powder form of extracts (Figure 4-3B).

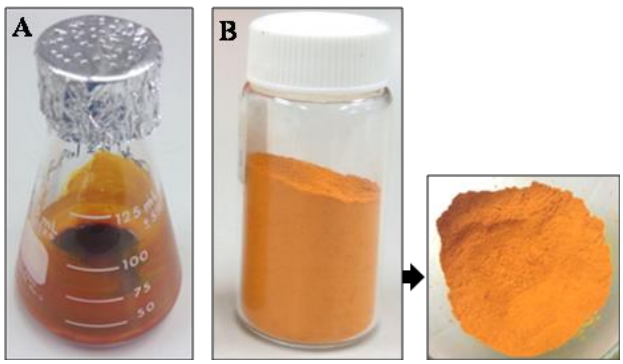

Figure 4-3 Physical appearances of crude ethanol *C. longa* extracts (**A**) and curcuminoid-rich *C. longa* extracts (**B**)

The Diaion® HP-20 column was a practical method for improving the curcuminoid content of *C. longa* extracts. This method can produce a CRE containing total curcuminoids

content up to 88.92% w/w dry weight extract, in which the amount of Cu I, Cu II, and Cu III were 72.81, 12.49, and 4.42% w/w, respectively. The other compounds including oleoresins and other pigments were also markedly excluded. Clearly, the Diaion® HP-20 was suitable for preparation of *C. longa* extracts enriched in curcuminoids. Therefore, a simple one-step purification method for preparation of the CRE involved a Diaion® HP-20 chromatographic column eluted with hydroalcoholic solutions, it was used only ethanol and water in our process. Thus, this method was an environmentally friendly process.

Table 4-7 Curcuminoids content in three batches of crude ethanol extract and Curcuminoid-rich *C. longa* extracts.

Extracts	Yield (%w/w)	Curcuminoids content% w/w			
		Cu I	Cu II	Cu III	Total
Ethanol extract	19.04	17.56 ± 0.33	5.34 ± 0.07	4.70 ± 0.14	27.60 ± 0.36[a]
CRE	3.40	72.81 ± 0.83	12.49 ± 0.57	4.24 ± 0.16	88.92 ± 0.70[b]

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.5 Phase solubility studies

The phase solubility diagrams of CRE in various concentrations of HP-β-CD are shown in Figure 4-4. The aqueous solubility of CRE was increased linearly with increasing HP-β-CD concentration over the entire concentration range studied, and this linear hose-guest correlation can be classified as A_L type according to Higuchi and Connors (1965). Because the straight line had a slope less than 1 in each compound, (i.e., 0.0074, 0.0202 and 0.0266 of Cu I, Cu II and Cu III respectively), the increase in solubility was due to the formation of 1:1 mole complex in solution with HP-β-CD. Moreover, the solubility of curcuminoids in HP-β-CD was significant increases compared to in the absence of HP-β-CD, indicating the solubilizing potential for all three curcuminoids by HP-β-CD.

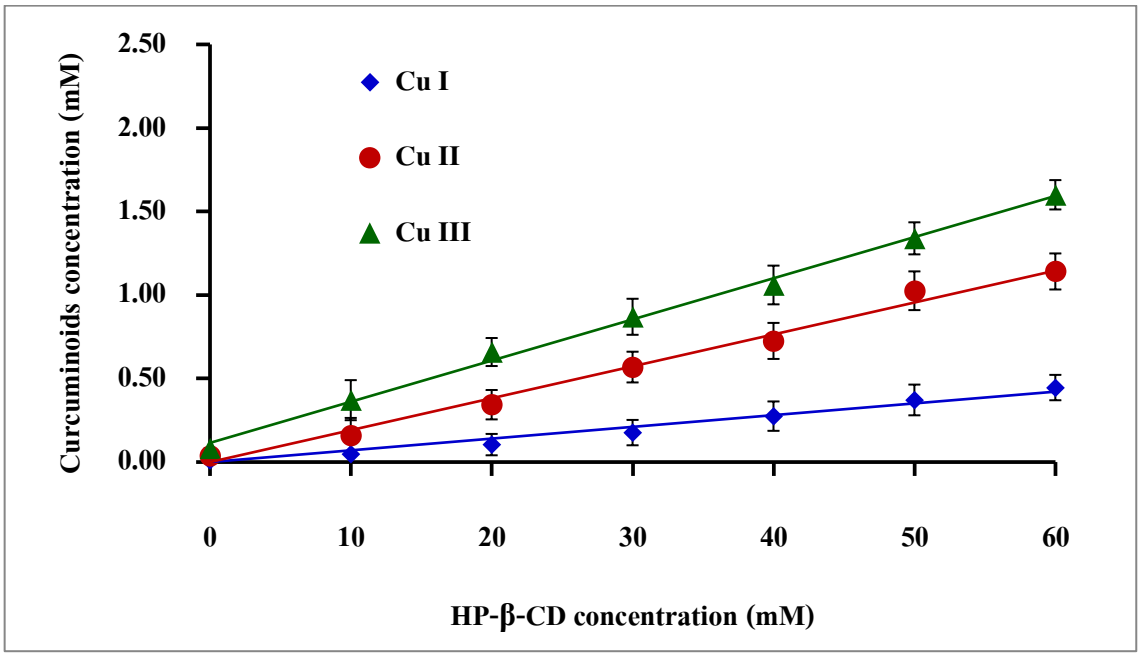

Figure 4-4 Phase-solubility diagrams of CRE/Hydroxypropyl-β-cyclodextrin

The effect of HP-β-CD on CRE water solubility and on the apparent stability constants of corresponding complexes is presented in Table 4-8. The Ks values of CRE/HP-β-CD inclusion complexes were found to be 473, 798 and 115 M^{-1} for Cu I, Cu II and Cu III respectively. According to Jasmina et al., (2012) only complexes with Ks between 100 and 1000 M^{-1} have industrial applications while Ks less than 100 M^{-1} represent an unstable drug-cyclodextrin system, whereas complexes with Ks value higher than 1000 M^{-1} could adversely affect drug absorption. In addition, a small Ks value indicates weak interaction; a larger value indicates the possibility of limited drug release from the complex. Therefore, the K_s values from this experiment indicate that the inclusion complex formed between curcuminoids and HP-β-CD were quite stable.

Table 4-8 Curcuminoids solubility (S_0), slope, correlation coefficient (R) diagrams stability constant (Ks) from the phase-solubility diagram.

Curcuminoids	S_0 (M^{-1})	Slope	R	Ks (M^{-1})
Cu I	0.0155	0.0074	0.9709	473.89
Cu II	0.0248	0.0202	0.9929	798.06
Cu III	0.1915	0.0266	0.9755	115.35

4.6 Preparation of CRE-cyclodextrin inclusion complex

4.6.1 Determination of molar ratios for preparation of CRE:HP-β-CD binary inclusion complex

Figure 4-5 shows water solubility of CRE from different ratios CRE: HP-β-CD of in binary inclusion complex and CRE noncomplex. The results show that an increase of HP-β-CD resulted in a significant increase in curcuminoids solubility. Similarly, Yatsu et al., (2013) reported that isoflavone compounds in soy isoflavone enrich fraction (SIF) was increased solubility in water when complexed with HP-β-CD. On the other hand, CRE noncomplex is insoluble in water. This result suggests that curcuminoids were included by HP-β-CD. Thus their aqueous solubility was improved. However, the increasing amounts of HP-β-CD higher than 1:1 molar ratio resulted in slight increase in CRE solubility compare to 1:1 molar ratio. Moreover, if a high amount of HP-β-CD is used, the cost of production and formulation bulk will increase (Loffson et al., 2002). Therefore, the suitable molar ratio (CRE: HP-β-CD) for preparation of binary inclusion complex was 1:1 (curcuminoids entrapment 90 %) and this ratio were used for further studies on the preparation of ternary inclusion complex. In addition, from solubility profile, we could predict the selectivity of curcuminoids with cyclodextrin. It was found that the solubility in water of Cu III > Cu II > Cu I respectively. These imply that besides the hydrophilic character or certain part; polarity and geometrical dimensions of the guest molecules also play an important role in the complex formation process (Astray et al., 2009).

4.6.2 Determination of PVP K30 concentration for preparation of CRE:HP-β-CD:PVP K30 ternary inclusion complex

Figure 4-6 shows the water solubility of CRE noncomplex and CRE ternary inclusion complex with differences % PVP K30 (F2-F4). The result exhibited that in ternary inclusion complexes showed higher water solubility than CRE noncomplex. In addition, a lower concentration of PVP K30 (F1 and F2), the lower solubilizing effect on curcuminoids, this may be attributed to the weak polymeric drug interaction (Yadav et al., 2009). However, formulation F3 (9% PVP K30) gave significantly highest solubility (72 μg/mL) when compared with all formulation. These results indicated that adding of hydrophilic polymer can increase the aqueous

solubility of curcuminoids from binary inclusion complex. This observation may be due to the improvement of wettability, hydrophilicity, the lowering of interfacial tension and solubilization effect of the polymer. Moreover, the aqueous solubility of curcuminoids (F1-F3) increases significantly which increasing concentration of polymer. This may be attributed to improved wetting of curcuminoids due to the formation of intermolecular of hydrogen bonding between curcuminoids and hydrophilic polymer (Savolainen et al., 1998). The similar result was reported by Jug et al., (2014) who revealed that the addition of PVP K30 into econazole nitrate/HP-β-CD inclusion complexes enhanced aqueous solubility of econazole nitrate about 6.7 fold. On the other hand, formulation F4 (PVP K30 11%) gave significantly lower solubility in water when compared with formulation F3. This may be due to the formation of an electrostatic bond between polymers which decreases their ability to form a complex (Yadav et al., 2012). Therefore, we selected ternary inclusion complexes with 9% PVP K30 (drug entrapment 90 %) for further study on the scale up preparation of ternary inclusion complexes.

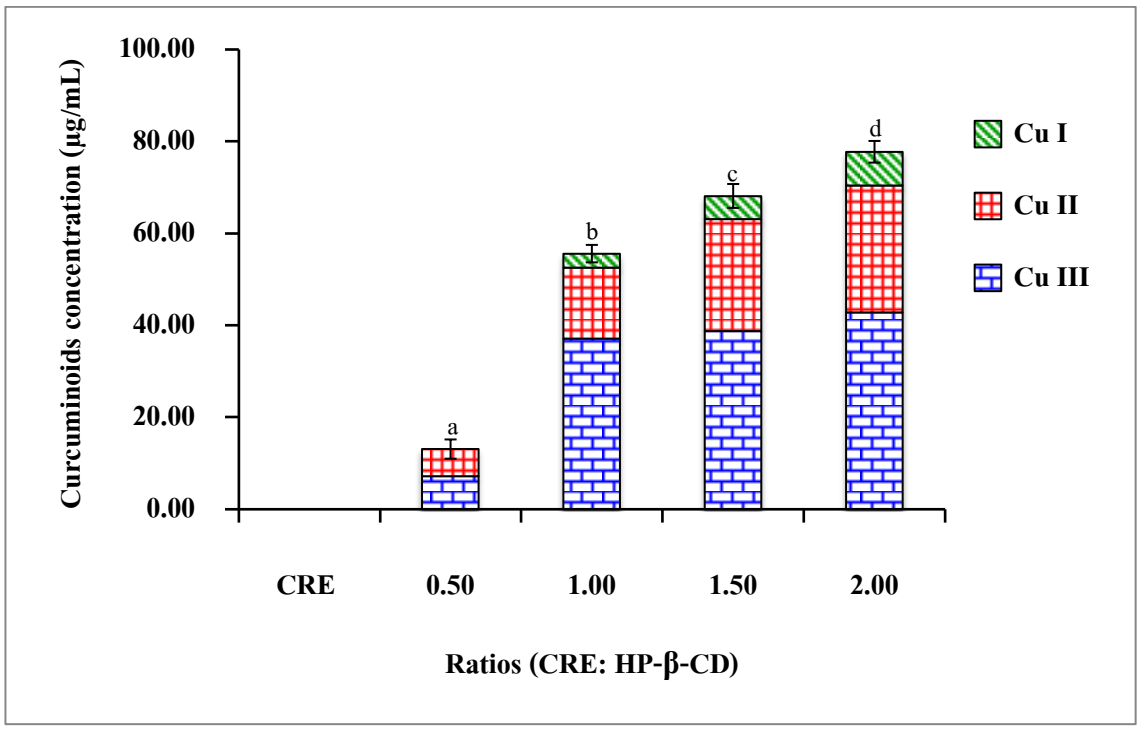

Figure 4-5 Solubility of CRE and difference ratios of CRE/HP-β-CD binary inclusion complexes in distilled water at 25 ± 1°C (mean ± SD., n=3)

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

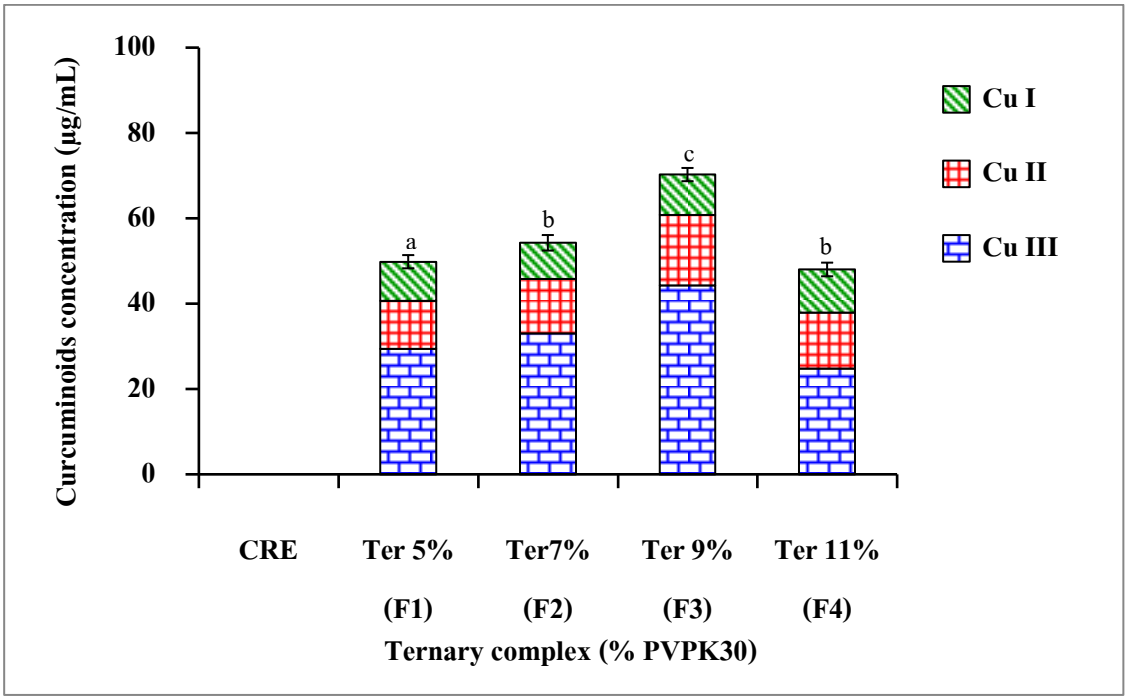

Figure 4-6 Solubility of CRE noncomplex, ternary inclusion complexes (CRE/HP-β-CD/PVP K30) with different weight ratios of PVP K30 in distilled water at 25 ± 1°C (mean ± SD., n=3)

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.7 Determination of reaction time for scale up preparation of binary inclusion complex

The CRE binary inclusion complex preparation using the optimal ratio from section 4.5.2 was performed determination of the reaction time (24, 48 and 72 h) for preparation of CRE binary inclusion complexes using 1:1 mole ratio. The result shown in Figure 4-7, with the lengthening of the reaction time, the solubility of curcuminoids increased. When the reaction time reached 48, the solubility of curcuminoids had the maximum solubility. However, the solubility of curcuminoids was not significantly increased with the extension of reaction time after 72 h compared to 48 h. According to Wang et al., (2014) was also found that long reaction time did not improve to physicochemical property (inclusion ratio) in soybean lacitin/β-CD inclusion complex. This may be due to the curcuminoids molecules are in rapid equilibrium complexed with HP-β-CD in the

solution (Loftsson et al., 2001). In addition, considering industrial scale application, less reaction time with efficient solubility is favorable. An appropriate reaction time for preparation CRE binary inclusion complexes using CRE: HP-β-CD (1:1 molar ratio) was therefore to be 48 h. The optimal condition was used for further studies on the scale-up preparation of CRE binary and ternary inclusion complexes.

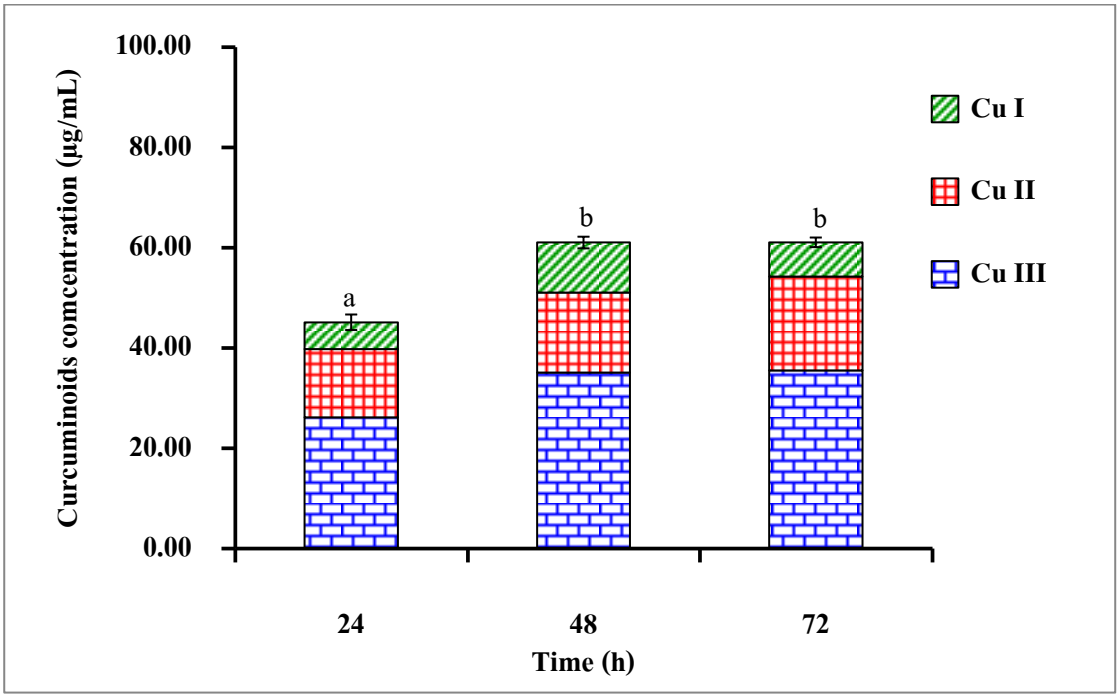

Figure 4-7 Effect of reaction times on solubility in distilled water at 25 ± 1°C of the inclusion complex of CRE with HP-β-CD (mean ± SD., n=3)

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.8 Scale-up production of CRE, binary and ternary inclusion complexes

A scale-up preparation of CRE, binary and ternary inclusion complexes were used the previously optimized conditions from Section 4.5. The Figure 4-8 was shown physical appearances in the solid and solution states of free CRE and binary and ternary inclusion complexes from scale-up preparation**.** The result shows that free CRE is orange powder form and binary and ternary inclusion complexes are also shown yellow powder form in the solid state.

Water solubility at 25 ± 1 °C of free CRE, binary and ternary inclusion complexes are shown in Figure 4-9. The solubility of free CRE was very low in water and precipitation when dissolved in water (Figure 4-8A). The solubility of total curcuminoids in binary and ternary inclusion complex was significantly ($p < 0.05$) greater than free CRE. The solubility value for binary and ternary inclusion complexes were 51 ± 1.31 and 75 ± 1.29 µg/mL, respectively. The solubility of curcuminoids from ternary inclusion complex was increased around two times when compared to CRE non complex. This result indicating that adding of hydrophobic polymers can increase the aqueous solubility of CRE from binary inclusion complexes (Jug et al., 2014). According to DSC and XRD results (Section 4.8), the amorphous properties of binary and ternary inclusion complexes were considered to be responsible for the solubility and dissolution enhancement. From this study, it was clearly concluded the ternary inclusion complex of CRE/HP-β-CD with PVP K30 was appropriately recommended for the preparation of more soluble complexes.

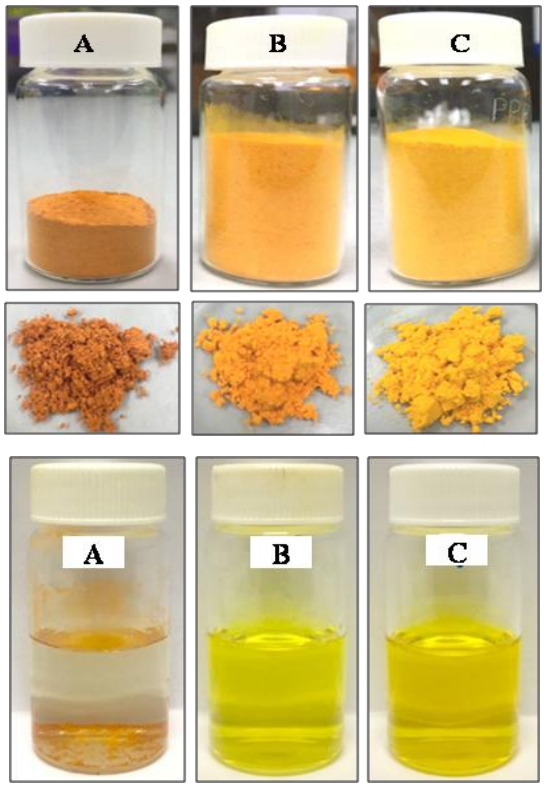

Figure 4-8 Physical appearances in solid and solution state of free CRE (**A**), binary inclusion complexes (CRE/HP-β-CD; B) and ternary inclusion complexes (CRE/HP-β-CD/ 9% PVP K30; **C**) prepared from scale up production.

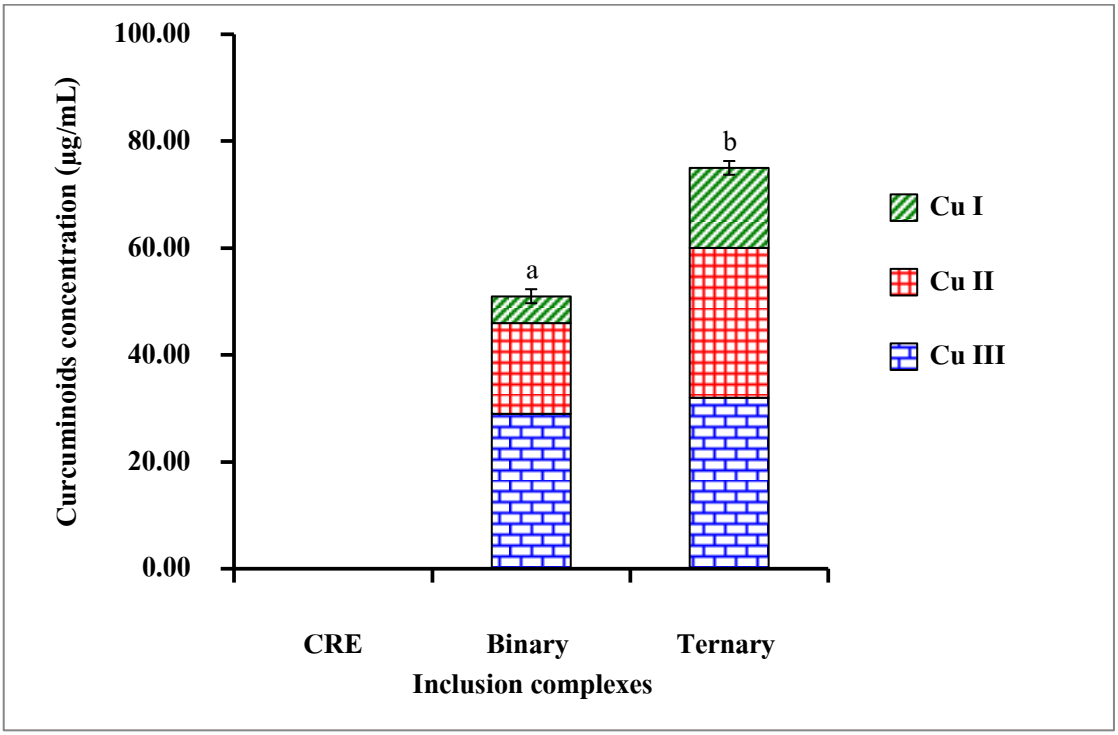

Figure 4-9 Solubility of curcuminoids in free CRE, binary (CRE/HP-β-CD) and ternary inclusion complexes (CRE/HP-β-CD/ 9% PVP K30) prepared from lot scale up production in distilled water at 25 ± 1°C (mean ± SD., n=3)

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

4.8 Characterization of CRE cyclodextrin inclusion complexes

4.8.1 Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of PVP K30, HP-β-CD, CRE, binary and ternary physical mixtures and binary and ternary inclusion complexes were shown in Figure 4-10. The FT-IR spectrum of CRE (Figure 4-10C) shows characteristic frequency band at 3507 cm^{-1} related to O-H stretching of phenol group and intra-molecular H bond frequency, 3406 cm^{-1} related to O-H stretching frequency, 1628 cm^{-1} related to C=O stretching frequency, 1600 cm^{-1} related to aromatic C=C stretching frequency (Mohan et al., 2012). The FTIR spectrum of PVP K30 (Figure 4-10A) showed characteristic frequency band 2955 cm^{-1} related to C-H stretching frequency, 1662 cm^{-1} related to C=O stretching frequency and 1292 cm^{-1} related to C-N stretching frequency (Geri et al.,

2011). The FTIR spectrum of HP-β-CD (Figure 4-10B) showed prominent peak at 3320 cm^{-1} related to O-H stretching, 2925 cm^{-1} related to C-H stretching, 1651 cm^{-1} related to H-O-H bending and 1034 cm^{-1} related to C-O, C-C, CCO, C-O-C stretching of glucose units (Mohan et al., 2012; Yao et al., 2014). The FTIR spectra of binary and ternary physical mixtures showed peaks corresponding to the original compounds. With respect to binary and ternary inclusion complexes (Figure 4-10E, 4-10G), the characteristic absorption band at 3507 cm^{-1}, 3406 cm^{-1} was found to have merged with board O-H peak of HP-β-CD present at 3415 cm^{-1}. This result may be due to the intermolecular hydrogen bonding between CRE, HP-β-CD and hydrophilic polymer (PVP K30). Moreover, the low intensity and slightly shift peak at 1628 cm^{-1} and 1600 cm^{-1} for binary and ternary inclusion complexes assumed that the aromatic ring ($C=C_{ring}$) of CRE might be entrapped inside the hydrophobic cavity of HP-β-CD, while another part may remain outside of HP-β-CD. Similarly, the phenomenon have been assumed that the spectral changes, at high frequency, in the O-H stretching profile, and, at low frequency, in the C-O, C-C, C-O-C, and C-O stretching vibrations could be attributed to the association, via H-bond, between the host and the guest during complexation process (Yatsu et al., 2013).

4.8.2 Powder X-ray diffractometry (PXRD)

The powder X-ray diffraction patterns of PVP K30, HP-β-CD, CRE, binary and ternary physical mixtures and binary and ternary inclusion complexes are shown in Figure 4-11. PXRD diffractogram of CRE was characterized by presence of several sharp peaks revealed a crystalline nature (Figure 4-11C), but PVP K30 and HP-β-CD were showed amorphous state (Figure 4-11A, 4-11B), these observations are consistent with the previous reports (Lokamatha et al., 2010; Yatsu et al., 2013; Vieira et al., 2015). The binary and ternary physical mixtures were presented peaks corresponding to the CRE (Figure 4-11D, 4-11F), demonstrating the presences of CRE in the crystalline state. Moreover, binary and ternary inclusion complexes diffractograms presented an absence of sharp peaks indicating the crystalline form of curcuminoid was changed to amorphous state (Figure 4-11E, 4-11G). Therefore, the complexes are in a totally different form and the curcuminoids are interacting with HP-β-CD probably by the formation of inclusion complexes (Yao et al., 2014).

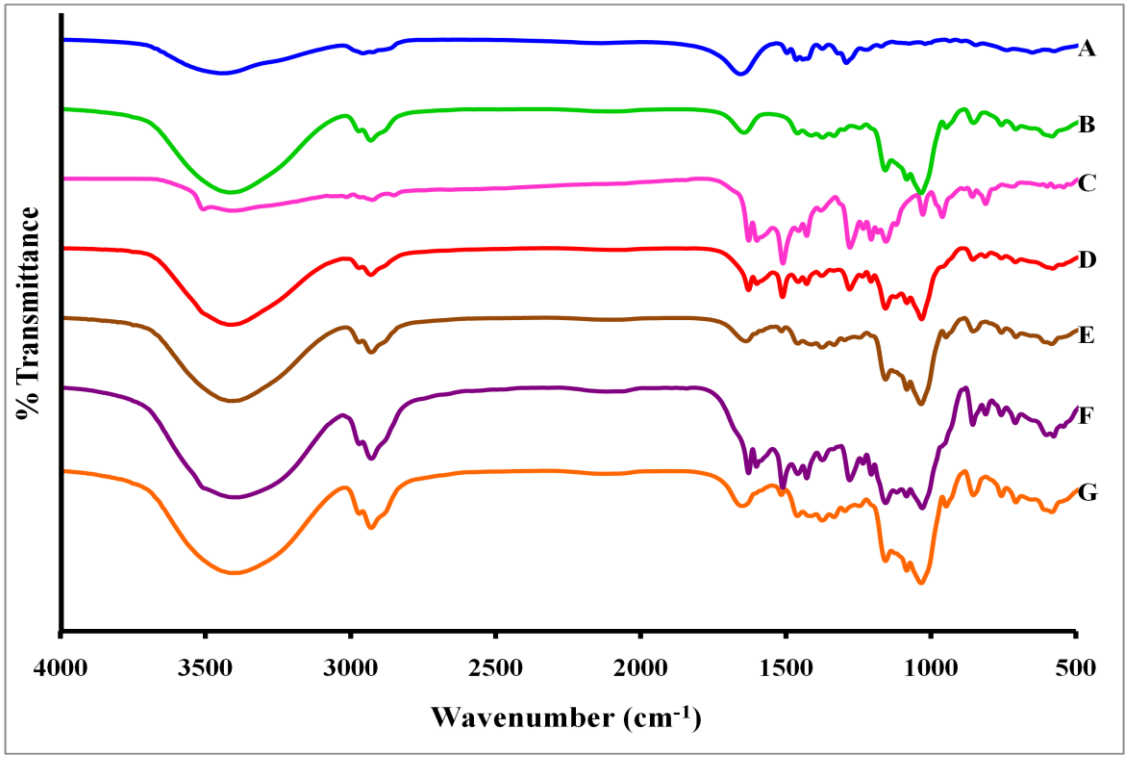

Figure 4-10 FTIR spectra of (A) PVP K30, (B) HP-β-CD, (C) CRE, (D) binary physical mixture, (E) binary inclusion complex (CRE/HP-β-CD), (F) ternary physical mixture and (G) ternary inclusion complex (CRE/HP-β-CD/PVP K30 9%).

4.8.3 Differential scanning calorimetry (DSC)

The DSC thermograms of PVP K30, HP-β-CD, CRE, binary and ternary physical mixtures and binary and ternary inclusion complexes are shown in Figure 4-12. CRE presents a broad endothermic peak at 170 ºC, indicating the melting point of CRE (Figure 4-12C). PVP K30 did not show any endothermic peak in the temperature range of study (Figure 4-12A), which may be due to the polymer relaxation during heating. This result indicated the amorphous form of polymer (Medarevic´ et al., 2015). The thermogram of HP-β-CD showed a broad endothermic peak of 334 ºC (Figure 4-12B). These observations are consistent with the previous reports (Yatsu et al., 2014; Deng et al., 2016). In the binary and ternary physical mixtures (Figure 4-12D, 4-12F), the intensity of the peak corresponding to the melting point of CRE is reduced, which were expected since the amount of CRE in physical mixture are very small, and the melting point of CRE in binary and ternary physical mixtures are shifted slightly, which may be due to interaction between CRE, HP-β-CD or polymer during the heating process and CRE remained crystalline

form (Singh et al., 2010). In addition, this result indicated that the simple mixing method is not enough to get true inclusion complexes (Medarevic´ et al., 2015). The binary and ternary inclusion complexes showed the complete disappearance of CRE peak (Figure 4-12C, 4-12G). The result indicated that the formation of amorphous inclusion complexes and true inclusion complexes might be obtained (Anwer et al., 2014).

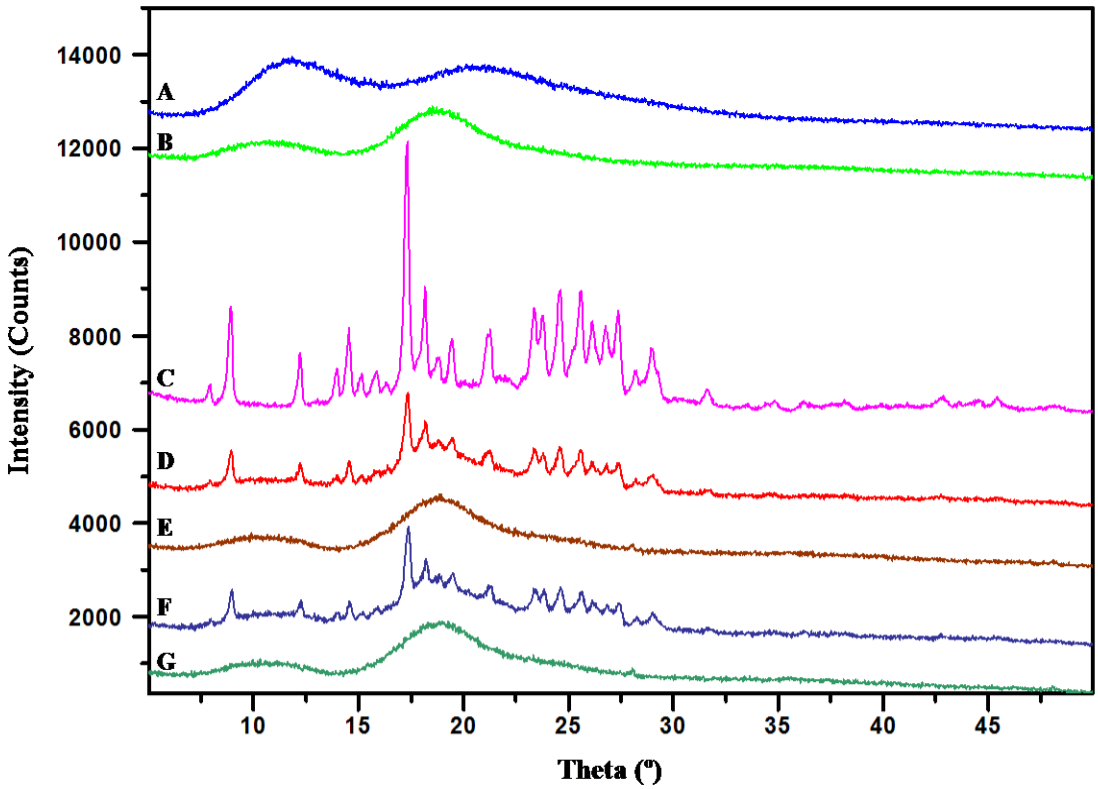

Figure 4-11 PXRD of (A) PVP K30, (B) HP-β-CD, (C) CRE, (D) binary physical mixture , (E) binary inclusion complex (CRE/HP-β-CD), (F) ternary physical mixture and (G) ternary inclusion complex (CRE/HP-β-CD/ 9% PVP K30).

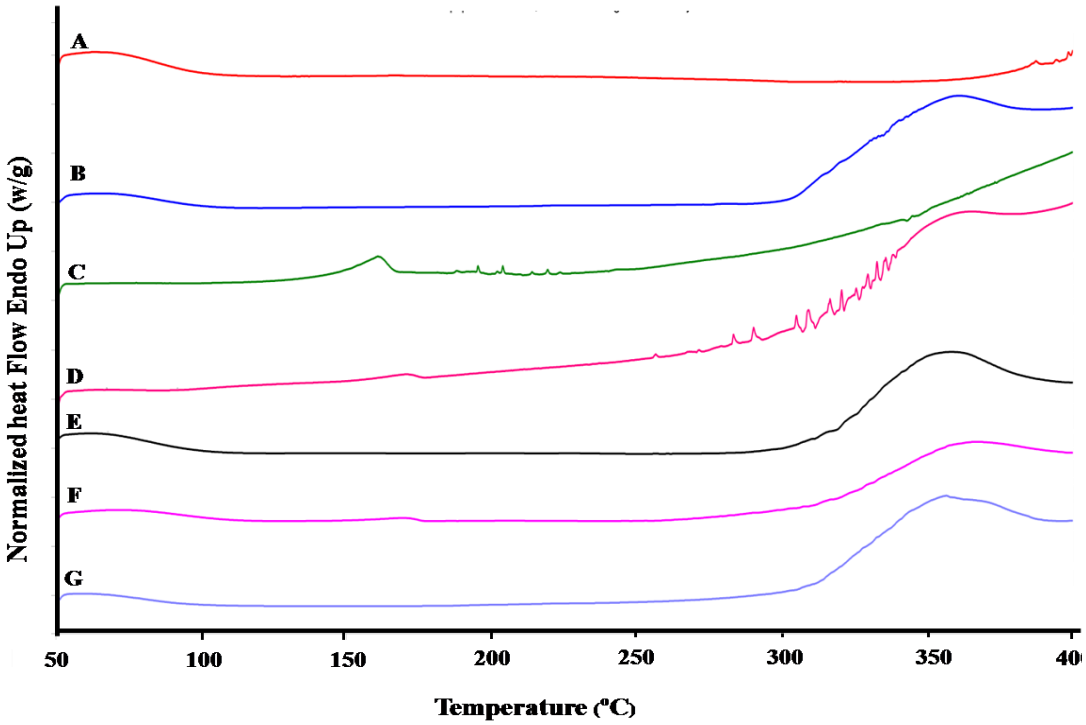

Figure 4-12 DSC thermograms of (A) PVP K30, (B) HP-β-CD, (C) CRE, (D) binary physical mixture , (E) binary inclusion complex (CRE/HP-β-CD), (F) ternary physical mixture and (G) ternary inclusion complex (CRE/HP-β-CD/PVP K30 9%).

4.8.4 Scanning electron microscopy (SEM)

The SEM photomicrographs of PVP K30, HP-β-CD, CRE, binary and ternary physical mixtures and binary and ternary inclusion complexes are shown in Figure 4-13. CRE shows a circle habit crystal and irregular surface (Figure 4-13C), and the typical structure of PVP K30 (Figure 4-13A) and HP-β-CD (Figure 4-13B) showed as amorphous or pieces of spherical shape, these observations are consistent with the previous reports (Vieira et al., 2015; Michalska et al., 2017). The binary and ternary physical mixtures showed both crystalline of CRE and amorphous polymer exists (Figure 4-13D, 4-13F), which indicated that no interaction takes place between the two or three components in the solid state. Binary and ternary inclusion complexes were completely differences from those of CRE, HP-β-CD or PVP K30. It was found to have amorphous powder with shard shape and broken edges appearance and also exhibited the reduction in CRE particle size (Figure 4-13E, 4-13G). These observations were in contract with the PXRD spectral analyses that host/guest complex take in the amorphous state, these results

were confirmed the formation of the inclusion complex of CRE and HP-β-CD (Yastu et al., 2013). Meanwhile, the reduced of CRE particle size, increased surface area and also close contact between polymer and CRE might be affected for increase solubility and dissolution rate of binary and ternary inclusion complexes (Lokamatha et al., 2010).

4.9 Dissolution study

The dissolution profiles of the binary and ternary inclusion complexes compared with CRE noncomplex are shown in Figure 4-14 and Figure 4-15. The dissolution of CRE noncomplex was very low at every point in two dissolution media. The hydrophobic property of the CRE prevented its contact with the dissolution medium causing it to float on the surface and consequently hindering its dissolution (Badr-Eldin et al., 2008). On the other hand, binary and ternary inclusion complexes have significantly increased in the dissolution of curcuminoids and provided a rapid release of curcuminoids up to 10 min in both media, when compared to CRE noncomplex. In addition, the ternary inclusion complex showed significant higher dissolution than the binary inclusion complex. However, the rate of dissolution of ternary inclusion complexes significant higher than binary inclusion complexes when compared at 10 min. This result may be due to the formation of hydrophilic polymer complexes sine polymers mainly interact with drug molecules via electrostatic bonds, i.e., ion-to-dipole and dipole-to-dipole bonds and other forces such as vander wals forces and hydrogen bridges (Yadav et al., 2009). Moreover, The other possible mechanisms of markedly increased in dissolution rate of curcuminoids from ternary inclusion complex may be due to solubilization effect of carrier, improved wettability and dispersibility, dissolution of hydrophilic carrier, reduction in particle size of drug, absence of aggregation of drug crystallites due to the conversion of the curcuminoids to amorphous state which leads to increases dissolution rate of poorly water-soluble curcuminoids (Figure 4-16). According to previous reports other drug/cyclodextrin ternary inclusion complexes, such as meloxicam/HP-β-CD/0.1% PVP K30 (El-Maradny et al., 2008), finasteride/HP-β-CD/0.1 % PVP K30 (Asbahr et al., 2009) and glyburide/HP-β-CD/ 20% PVP K30 (Zoeller et al., 2012) have also show enhance solubility and dissolution rate of hydrophobic drug.

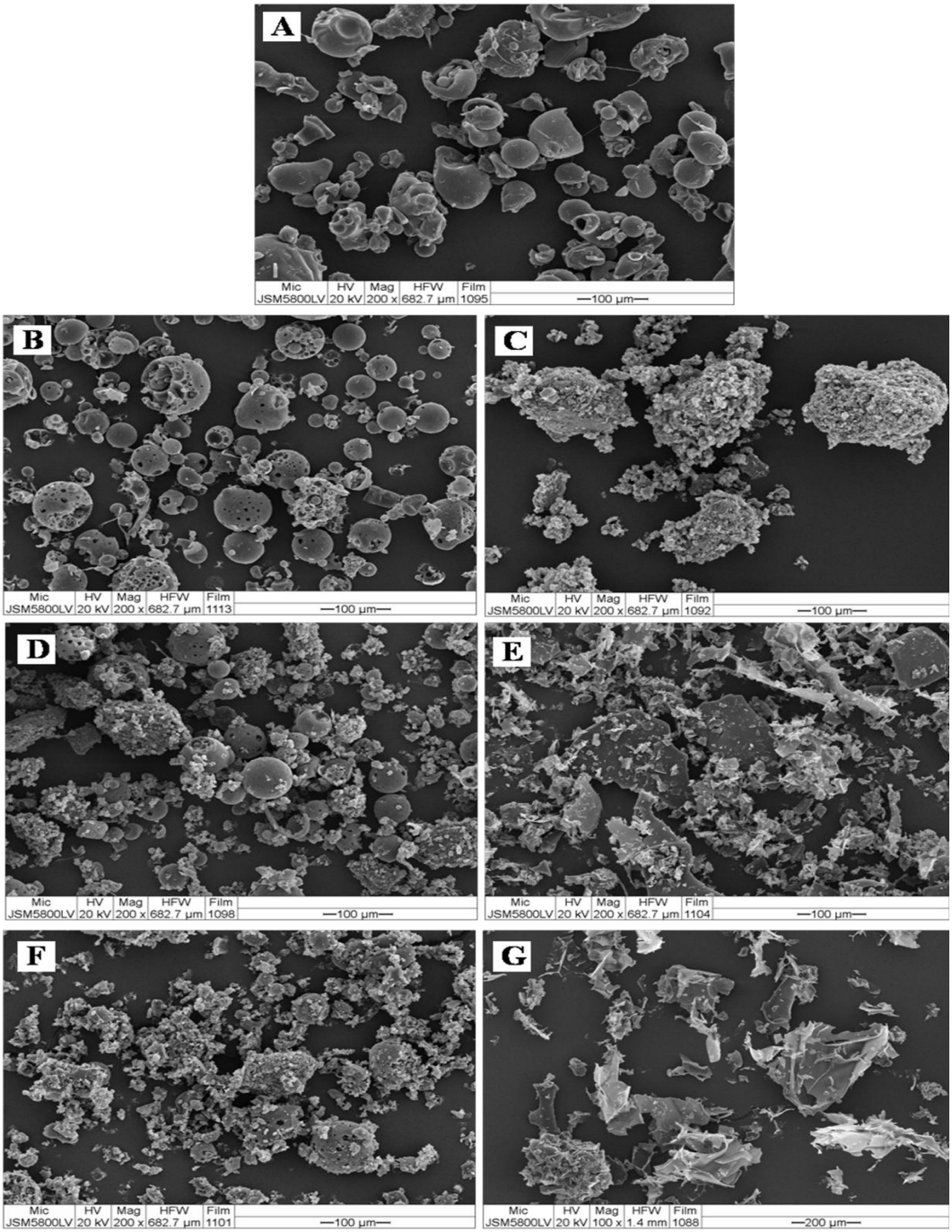

Figure 4-13 Scanning electron microphotographs of (A) PVP K30, (B) HP-β-CD, (C) CRE, (D) binary physical mixture , (E) binary inclusion complex (CRE/HP-β-CD), (F) ternary physical mixture and (g) ternary inclusion complex (CRE/HP-β-CD/ 9% PVP K30).

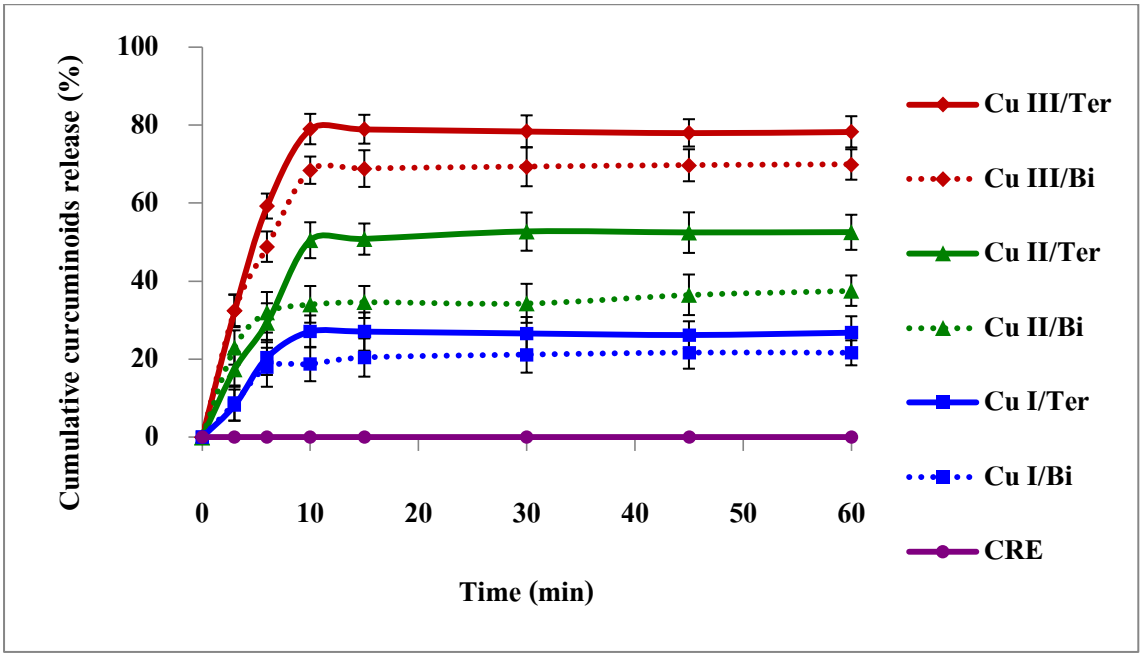

Figure 4-14 Dissolution profiles of curcuminoids in CRE, binary inclusion complex (CRE/HP-β-CD) and ternary inclusion complex (CRE/HP-β-CD/9% PVP K30) in SGF at 37 ± 0.5°C (mean ± SD., n=3)

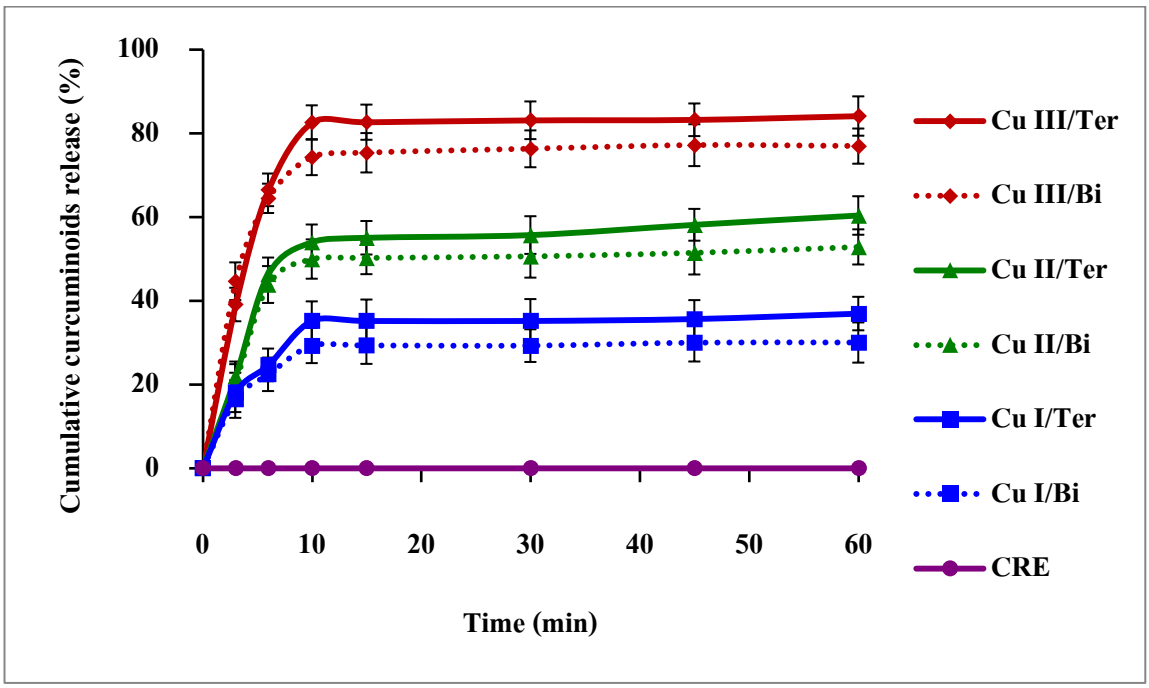

Figure 4-15 Dissolution profiles of curcuminoids in CRE, binary inclusion complex (CRE/HP-β-CD) and ternary inclusion complex (CRE/HP-β-CD/9% PVP K30) in SIF at 37 ± 0.5°C (mean ± SD., n=3

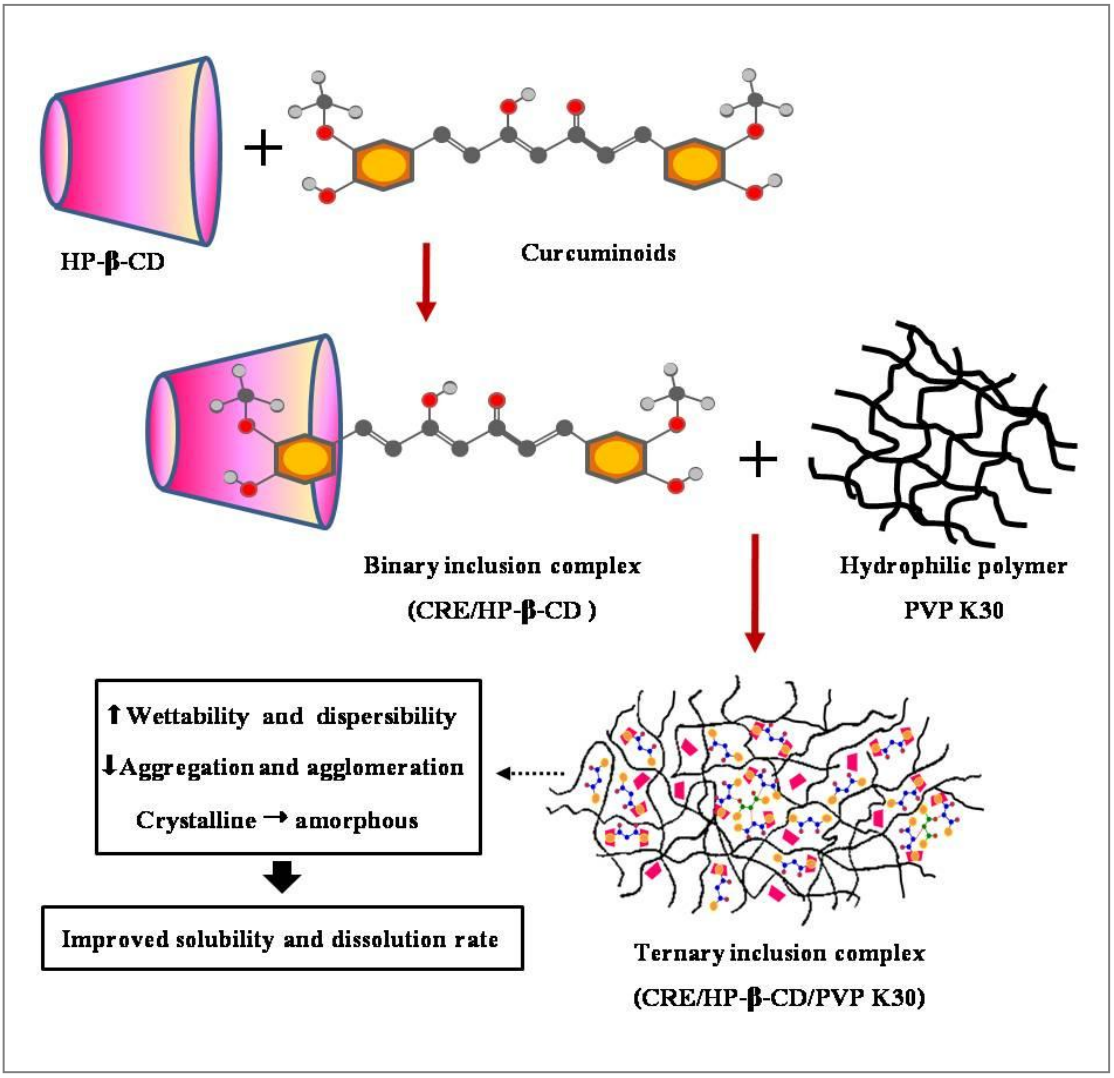

Figure 4-16 The possible mechanism of solubility and dissolution enhancement of CRE ternary inclusion complexes were adapted from Savolainen et al., (1998); Loffson et al.,(2002); Yadav et al., (2012).

4.10 Stability study

4.10.1 Effect of temperature on the stability of the extracts and binary and ternary inclusion complexes

The effect of temperature on the stability of CRE and binary and ternary inclusion complexes were investigated under two temperature including 4°C and 25°C, by kept in the container that protected from light. The CRE and binary and ternary inclusion complexes are

physically and chemically stable for at least 4 months when stored at 4°C and 25°C (Figure 4-17 and Figure 4-18). Similarly, Paramera et al., (2011) and Mangolim et al., (2014) reported that curcumin and curcumin-β-cyclodextrin inclusion complex were stable when kept at 4°C and 25°C in the dark condition. These result suggested that CRE and binary and ternary inclusion complexes were stable when kept in a cool place, i.e. between 4 - 25°C, under dark condition.

4.10.2 Effect of accelerated condition on the stability of the extracts and binary and ternary inclusion complexes

A stability test of CRE binary and ternary inclusion complexes under accelerated condition was carried using a stability chamber at 45°C and 75% RH, and protected from light. The result showed that the physical appearance did not change for at least 4 months. Moreover, the content of total curcuminoids in the CRE and binary and ternary inclusion complexes were found to be insignificantly different (>90% w/w) after kept for four months (Figure 4-19). Therefore, the result suggests that accelerated condition does not affect the stability of the CRE and binary and ternary inclusion complexes. However, further formulation of developing products and longer stability should proceed.

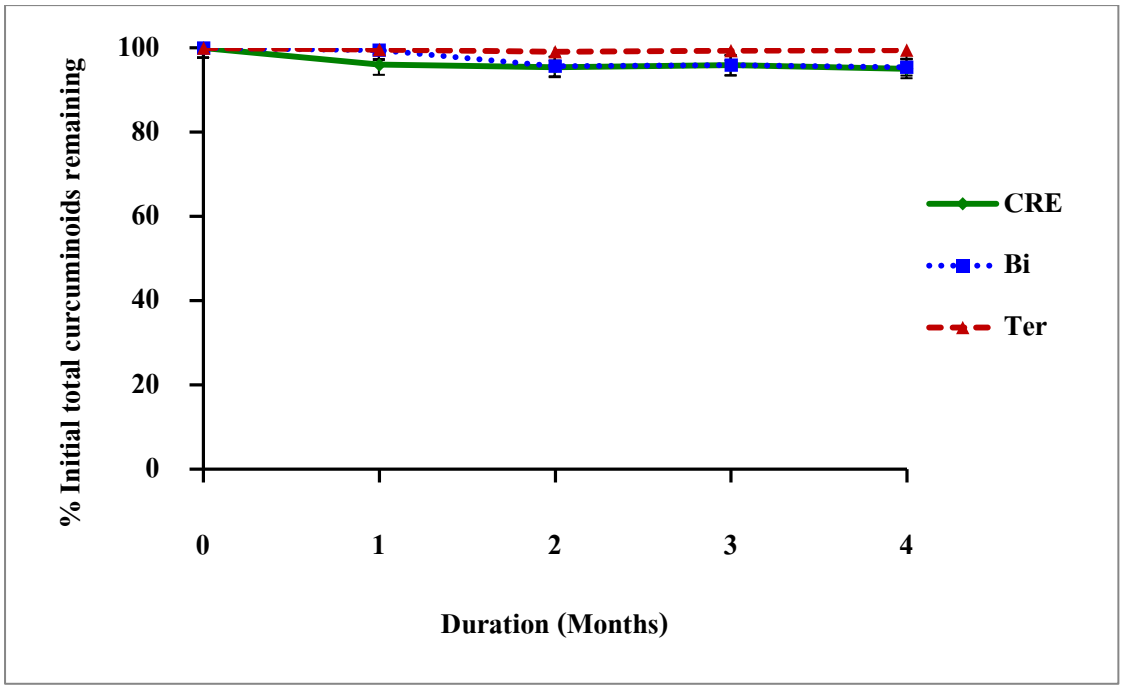

Figure 4-17 Stability of CRE, binary inclusion complex (CRE/HP-β-CD) and ternary inclusion complex (CRE/HP-β-CD/9% PVP K30) in effect of temperature 4 ± 1°C (mean ± SD., n=3)

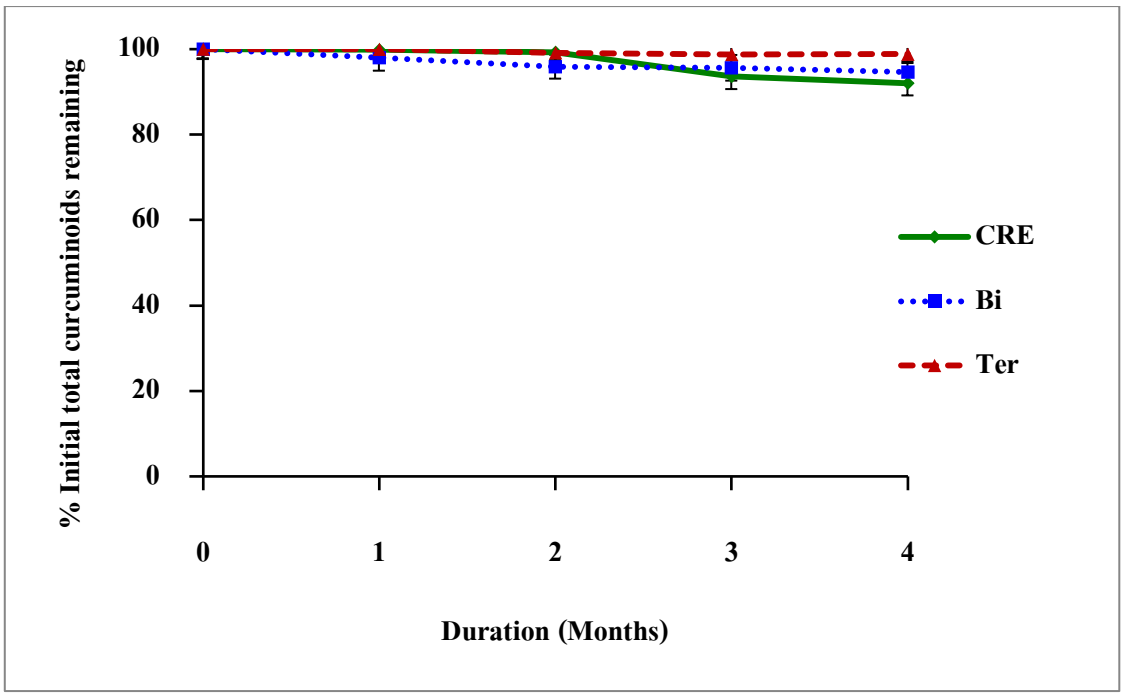

Figure 4-18 Stability of CRE, binary inclusion complex (CRE/HP-β-CD) and ternary inclusion complex (CRE/HP-β-CD/9% PVP K30) in effect of temperature 25 ± 1°C (mean ± SD., n=3)

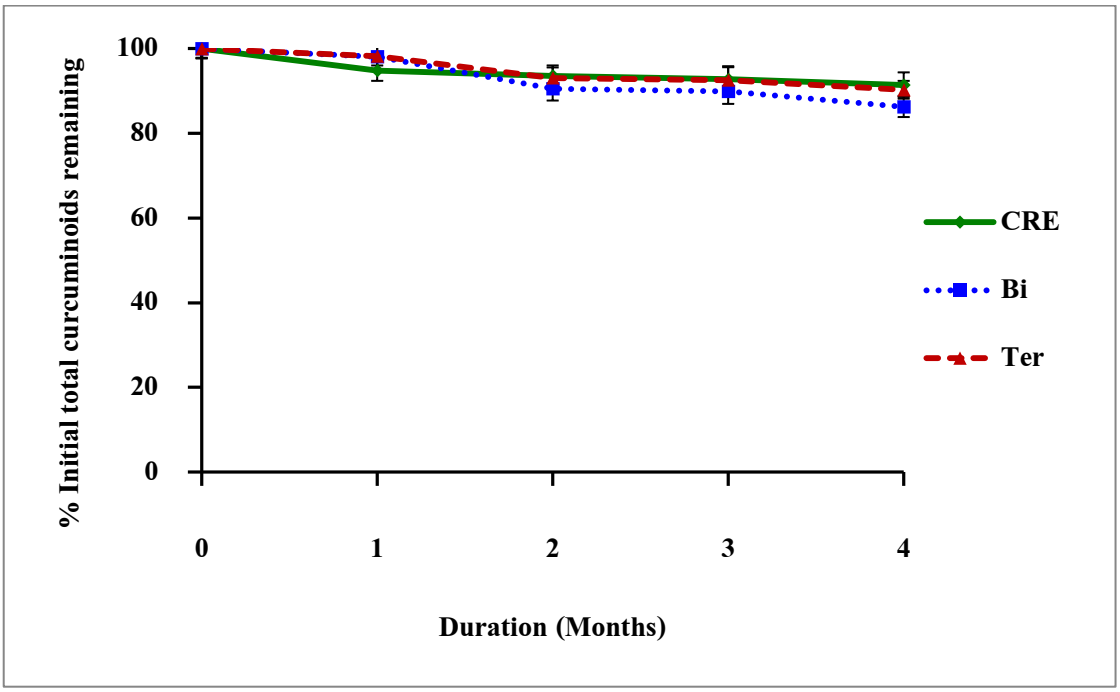

Figure 4-19 Stability of CRE, binary inclusion complex (CRE/HP-β-CD) and ternary inclusion complex (CRE/HP-β-CD/9% PVP K30) in effect of accelerated condition (45 ± 1ºC, 75 ± 5 % RH) (mean ± SD., n=3)

4.11 *In vitro* anti-cancer activity

In this study, SRB assays were used to evaluate the cell cytotoxicity of CRE binary and ternary inclusion complex. The SRB assay used for cell density determination, based on the measurement of SRB to bind cellular protein component (Vichai & Kirtikara, 2006). The cytotoxic activities of pure curcuminoids compound, crude ethanol extract, CRE from *C. longa* were evaluated in four human cancer cell lines, which are A-549, MCF-7, HeLa and HT-29 cell lines the result showed that pure curcuminoids compound exhibited higher cytotoxic effect than the crude ethanol extract, In addition, the CRE showed good anticancer activity than the crude ethanol in all human cancer cell lines with the IC_{50} values 5.18, 3.46, 2.73 and 7.66 µg/ml of A-549, MCF-7, HT-29 and HeLa cell lines respectively (Table 4-9).

Table 4-9 Cytotoxic activity expressed as IC_{50} (µg/mL) of Cu I, CuII, Cu III, Crude ethanol and CRE against A-549, MCF-7, HT-29 and HeLa cell lines.

Extract/complexes	IC_{50} (µg/mL)			
	A-549	MCF-7	HT-29	HeLa
Cu I	2.96 ± 0.43^{a}	3.39 ± 0.13^{a}	4.36 ± 0.18^{a}	7.45 ± 0.42^{a}
Cu II	2.62 ± 0.15^{a}	3.06 ± 0.14^{b}	2.77 ± 0.13^{b}	6.21 ± 0.22^{b}
Cu III	2.79 ± 0.09^{a}	2.37 ± 0.17^{c}	3.06 ± 0.22^{c}	5.95 ± 0.07^{c}
Crude EtOH	12.62 ± 0.51^{b}	12.95 ± 0.64^{d}	7.57 ± 0.30^{d}	12.23 ± 0.39^{d}
CRE	5.18 ± 0.39^{c}	3.46 ± 0.37^{a}	2.73 ± 0.25^{b}	7.66 ± 0.13^{a}
Camptothecin	< 0.02	< 0.02	< 0.02	< 0.02

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different ($P < 0.05$) when compared to means.

Base on the IC_{50} value on cytotoxic effect on the human cancer cell, the CRE exhibited stronger anticancer activity than the crude ethanol extracts, on all cancer cell lines. This implies that an increase of curcuminoids content in the extract was increasing cytotoxicity. Comparison of cytotoxic results revealed that the pure curcuminoids were relatively active in all human cancer cell lines. The IC_{50} values ranged from 2.62-7.45 µg/ml. This compound has also been reported to show anticancer activity in various cancer cell lines (Basile et al., 2009). Compound Cu I, Cu II and Cu III exhibited higher effect than crude extracts. This result is concurrent with the previous

study on the anticancer effect of curcuminoids from *C. longa*. Recently, many reports have offered scientific data that curcuminoids produce anticancer effects over multiple mechanisms *in vitro* anticancer model (Palve et al., 2012; Perrone et al., 2015). Especially, curcumin has been reported to suppress proliferation and induce apoptosis in a wide variety of cancer cells via extensive signaling pathway (Karunagaran et al., 2005; Tuorkey, 2014).

The effects of CRE binary and ternary inclusion complex and free CRE on A549, HeLa and HT-29 cells cytotoxicity were illustrated in Table 4-10. The result showed that cytotoxic effects of CRE binary and ternary inclusion complexes on all cancer cell lines were greater than free CRE. This may be because of the improved solubility and release of curcuminoids in binary and ternary inclusion complexes, wherewith curcuminoids in aqueous solution promoted the cellular uptake of curcuminoids molecules into the cell and act (Tan et al., 2012). According to previous reports other hydrophobic compounds, such as anthraquinones in *Rheum rhabarbarum* extracts (Hsu et al., 2013) and ferulic acid, ligustilide and butylidenephthalide in *Angelica sinensis* extracts (Hsu et al., 2014) have also show enhance the anticancer effect and target compounds cellular uptake when complete with HP-β-CD.

Table 4-10 Cytotoxic activities (IC_{50} μg/mL) of free CRE, binary and ternary inclusion complexes against human cancer cell lines.

Extract/complexes	IC_{50} (μg/mL)		
	A-549	HT-29	HeLa
CRE	5.18 ± 0.39^{a}	4.11 ± 0.12^{a}	7.69 ± 0.13^{a}
Binary inclusion complex	4.03 ± 0.18^{b}	3.42 ± 0.57^{b}	6.57 ± 0.27^{b}
Ternary inclusion complex	3.14 ± 0.20^{c}	2.58 ± 0.61^{c}	5.47 ± 0.17^{c}
Camptothecin	< 0.02	< 0.02	< 0.02

Means followed by the same letter within a column are not significantly different according to Tukey's test. Significantly different (P<0.05) when compared to means.

CHAPTER 5

CONCLUSIONS

From this research work the following conclusions can be drawn:

1. This study demonstrated the potential of using pharmaceutical excipients i.e. propylene glycol, polyethylene glycol 400, glycerin and ethanol as alternative solvents for extraction of curcuminoids from *C. longa* powder. The ethanol proceeds higher efficiency and selectivity for curcuminoids extraction.

2. Microwave-assisted extraction (MAE) was recommended green extraction method that can be applied for an extraction of curcuminoids from *C. longa* powders. The optimal conditions of MAE were: using ethanol as solvent, irradiation power 180 W, 75 ºC, power on 30 sec, and one extraction times. In addition, the most suitable MAE for larger scale extraction (240 g powders and 2.4 L solvent) using ethanol were irradiation power 900 W, 75ºC, three irradiation cycles, and one extraction times. Under the optimal MAE conditions, the total curcuminoids, curcumin (Cu I), demethoxycurcumin (Cu II) and bisdemethoxycurcumin (Cu III) were 64.2, 15.4, and 15.6 mg/g, respectively.

3. A simple purification method can be used for the preparation of the curcuminoids-rich extract (CRE) involving a Diaion® HP-20 chromatographic column eluted with 55% and 60% ethanol. CRE used for the further study was standardized to contain total curcuminoids content not less than 88% w/w dry weigh. This process is environmental friendly.

4. CRE was showed higher anticancer activity against MCF7, HeLa, A-549 and HT-29 cell lines and potential use of CRE for anticancer proposes in nutraceutical and food applications. Respecting an industrial application, CRE has more expediency than the use of pure curcuminoids in terms of a simple, low-cost production process and environment-friendly process.

5. For increased water-solubility of curcuminoids in CRE by the formulation of CRE-cyclodextrin inclusion complexes. The optimal condition were; CRE and HPBCD 1:1 mole ratio, stirred at room temperature for 48 h, 9% PVP K30.
6. The binary and ternary inclusion complexes have significantly increased in the dissolution of curcuminoids. The CRE inclusion complexes were stable for 4 months when kept in well-closed and accelerated conditions at 45°C, 75% humidity.
7. The binary and ternary inclusion complexes were showed significantly higher anticancer activity than CRE against A-549, HT-29 and HeLa cell lines and potential use of inclusion complexes for anticancer proposes in pharmaceuticals industry application.

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Patents

ภาคภูมิ พาณิชยูปการนันท์ และ ลิขิต ลาเต๊ะ. 2558. สิทธิบัตรเรื่องกรรมวิธีการเตรียมสารสกัดขมิ้นชันที่มีสารเคอร์คิวมินอยด์. เลขที่คำขอ 1601001174 ลงวันที่ 2 มีนาคม 2559.

ภาคภูมิ พาณิชยูปการนันท์ ลิขิต ลาเต๊ะ และ นัฏฐา แก้วนพรัตน์.2559. อนุสิทธิบัตรเรื่อง กรรมวิธีการเพิ่มการละลายน้ำของสารสกัดเคอร์คูมินอยด์โดยการเตรียมเป็นสารประกอบเชิงซ้อนและผลิตภัณฑ์ที่ได้จากกรรมวิธีดังกล่าว 1703001404 ลงวันที่ 2 สิงหาคม 2560.