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Enhancement of Dissolution and Antioxidant Activity of Kaempferol Using a Nanoparticle Engineering Process

Cheng-Wei Tzeng,[†] Feng-Lin Yen,[‡] Tzu-Hui Wu,[∥] Horng-Huey Ko,[‡] Chiang-Wen Lee,[⊥] Wen-Sheng Tzeng,[*,#] and Chun-Ching Lin[*,†,§]

[†]Graduate Institute of Natural Products, [‡]Department of Fragrance and Cosmetic Science, and [§]School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan

[∥]Health Bureau of Kaohsiung County Government, Kaohsiung, Taiwan

[⊥]Department of Nursing, Division of Basic Medical Sciences, and Chronic Diseases and Health Promotion Research Center, Chang Gung Institute of Technology, Chia-Yi, Taiwan

[#]Attending Diagnostic Radiologist, Chi-Mei Foundation Hospital, Tainan, Taiwan

ABSTRACT: Kaempferol (KAE) is a strong antioxidant flavonoid compound, but its clinical application is limited by quantity and poor dissolution property. However, the dissolution mechanism of a kaempferol nanoparticle formulation (KAEN) has not yet been elucidated. The aim of the present study was therefore to use a nanoparticle engineering process to resolve the dissolution problem. Our data indicated that KAEN effectively increased the dissolution percentage by particle size reduction, high encapsulation efficiency, amorphous transformation, and hydrogen-bond formation with excipients. In addition, we used several different antioxidant activity assays to evaluate KAE and KAEN. The data indicated that KAEN retained potent antioxidant activity after the nanoparticle engineering process and showed better antioxidant activity than KAE dissolved in water ($P < 0.05$). According to these findings, we concluded that KAEN could be a low-dose alternative to KAE in health food and future clinical research.

KEYWORDS: Kaempferol, nanoparticle engineering process, dissolution, antioxidant activity

INTRODUCTION

Dietary polyphenols, such as flavonoids and phenolic acid, could effectively prevent the aggravating effect the modern human diet has on many diseases.[1] Kaempferol (3,4′,5,7-tetrahydroxyflavone; KAE; Figure 1), a well-known flavonoid compound, is widely distributed in natural foods and plants, such as onion,[2] kale,[3] and endive.[4] Previous studies have demonstrated KAE's pharmacological activities, including antioxidant,[5] antivirus,[6] anti-inflammation,[7] antiallergic,[8] and anticancer properties.[9]

Mallick et al. reported that poor water solubility and dissolution of compounds influenced their bioavailability.[10] However, KAE was purchased from Sigma-Aldrich Co., and its chemical information reported that the solubility of KAE was 20 mg/mL in ethanol and 50 mg/mL in DMSO, respectively. Barve et al. have reported that KAE converted to other active compounds by phase I oxidative metabolism and phase II glucuronidation in the liver and intestine, such as quercetin and isorhamnetin. These factors led to the oral administration with 100 or 250 mg/kg of KAE, which had very low bioavailability (2%) in a rat model.[11] These drawbacks restricted its application in clinical medicine even though KAE possesses many pharmacological activities. Currently, many molecular biological studies have used dimethyl sulfoxide (DMSO) or ethanol to resolve the poor water solubility of hydrophobic compounds, such as quercetin[12] and curcumin.[13] However, higher concentrations of DMSO and ethanol are questionable due to their risk for neurological toxicity[14] and hepatotoxicity,[15] respectively.

Ratnam et al. have reported that delivery systems are applicable in overcoming these drawbacks of hydrophobic compounds to significantly increase the effectiveness of drug and food materials and to decrease the dosage required, such as nanoparticle and liposome.[16] Nanoparticle systems, stable colloidal particle systems that range in size from 10 to 1000 nm, have improved material's poor aqueous solubility, dissolution, and/or bioavailability, such as has been shown for curcumin[17] and silybin.[18] As a size reduction method, the nanoparticle engineering process (NPS) is one of the excellent drug delivery systems. The use of NPS has effectively improved in vitro dissolution of many poor water-soluble drugs, such as nanoprecipitation, wet milling, and high pressure homogenization.[19]

Nontoxic excipients have been used instead to prepare many nanoparticle formulations. Eudragit E100 (EE100) is a cationic polymer consisting of a 1:2:1 ratio of butyl methacrylate, dimethylaminoethyl methacrylate, and methyl methacrylate. EE100 has commonly been used in oral pharmaceutical formulation due to its nontoxicity and edibility, such as tablet and solution.[20,21] In addition, polyvinyl alcohol is generally regarded as a nontoxic excipient, and it also is commonly used as a stabilizer in the pharmaceutical formulation. The aim of the present study was to develop a novel kaempferol nanoparticle system (KAEN) using a simple and quick nanoparticle engineering process with EE100 and PVA as excipients. However, Dickinson et al. have reported that a dissolution study of a new drug formulation should be carried out before the determination of bioavailability in clinical trial.[22] Interestingly, the mechanism of dissolution of KAEN has

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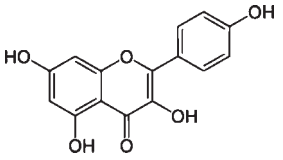

Figure 1. Chemical structure of kaempferol.

not yet been reported. We investigated several physicochemical characterizations of KAE and KAEN to elucidate the dissolution mechanism, such as the determination of particle size, the amorphous transformation, hydrogen-bond formation, and in vitro drug release. However, the antioxidant activity of KAE was observed to decline as a result of pharmaceutical preparation. We ran several antioxidant assays to confirm the decreased antioxidant activity of KAEN and compared them to the activity KAE.

MATERIALS AND METHODS

Chemicals and Reagents. Kaempferol, polyvinyl alcohol (PVA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO). Sodium dihydrogen phosphate (NaH_2PO_4) and sodium phosphate (Na_2HPO_4) were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Aminoalkyl methacrylate copolymers (Eudragit E100; EE100) were obtained from Röhm Pharma (Dramstadt, Germany). All other chemical regents were of analytical grade.

Kaempferol Nanoparticle Systems Prepared by the Nanoparticle Engineering Process. The kaempferol nanoparticle systems with a ratio of KAE:EE:PVA of 1:10:10 (w/w/w) were prepared by modified nanoprecipitation method from Bilati et al.[23] Briefly, 200 mg of EE100 was added to 10 mL of ethanol and dissolved in an ultrasonic water bath (Branson, U.S.) at 37 °C. Next, 20 mg of kaempferol was added to the organic solution. The organic phase solution was then quickly injected into a 30 mL external aqueous solution containing 200 mg of PVA and homogenized at 22 000 rpm for 25 min. Subsequently, the ethanol was removed by rotary vacuum evaporation in a 40 °C water bath. The remaining solution, the kaempferol nanoparticle solution (KAEN), was approximately 18 mL in volume and was stored in a refrigerator until being used for antioxidant activity assays. Some of the KAEN was lyophilized with a freeze-dryer, and the lyophilized powder was collected and stored in a moisture-proof container until characterization of its physicochemical properties. Additionally, a physical mixture containing the same ratios of KAE:EE100:PVA (1:10:10; w/w/w) was mixed and ground with a mortar for comparison of physicochemical characterization.

Determination of Particle Size and Morphology of KAEN. A N5 submicrometer particle size analyzer (Beckman Coulter, U.S.) containing a 25 mW helium−neon laser lamp with a wavelength of 632.8 nm was used for photon correlation spectroscopy to determine the mean particle size. The uniformity of the particle sizes was related to the polydispersity index (PI) of the analyzer. For analysis, the KAEN solution was diluted 10-fold with distilled water and placed into a 1 mL cuvette for analysis. Each value was measured in triplicate, and the results are reported as the mean ± standard distribution. In addition, a transmission electron microscope (JEOL JEM-2000 EXII TEM, Tokyo, Japan) was used to examine the morphology of the KAEN. Before examination, the sample was stained with 0.5% (w/v) phosphotungstic acid and fixed on a copper grid.

Determination of the Amorphous Transformation. Differential scanning calorimetry (DSC) and X-ray diffractometry (XRD) were used to determine the crystal polymorphism of KAE and KAEN. Briefly, approximately 3 mg of KAE, KAEN, and their physical mixture was placed into aluminum pans and then put into the DSC (Perkin-Elmer, Norwalk, CT). The temperature of scanning was set at 150 °C, the scanning rate was 10 °C min^{-1}, and the final temperature was 300 °C. In addition, XRD (Siemens D5000, Germany) with Ni-filtered Cu Kα radiation was used to determine the crystalline pattern of pure KAE, lyophilized KAEN, and their physical mixture. XRD was performed at 40 kV and 25 mA. The scanned angle setting was over the 2θ interval from 2° to 50°, and the scan rate was 1°/min. DSC and XRD determinations were made in triplicate.

Determination of Hydrogen-Bond Formation. The spectra of Fourier transform infrared spectroscopy (FT-IR) and ^1H nuclear magnetic resonance (^1H NMR) analyses were used to elucidate hydrogen-bond formation between kaempferol and polymers. The FT-IR spectra of pure kaempferol, KAEN, and their physical mixture were obtained on a Perkin-Elmer 2000 spectrophotometer (Perkin-Elmer, Norwalk, CT). Briefly, each sample was mixed with potassium bromide and ground to powder in an agate mortar. Each powder was then compressed into thin tablets for FT-IR spectroscopy. The scanning was set from 400 to 4000 cm^{-1}, and the resolution was 1 cm^{-1}. Each sample was measured and recorded in triplicate. In addition, the ^1H NMR spectra of KAE and KAEN were recorded using a Varian Mercury Plus 400 Actively Shielded NMR System (Oxford Instrument Co., Abingdon, Oxfordshire, U.K.). Five milligrams of each sample was dissolved in 0.8 mL of DMSO-d_6, and each sample was measured and recorded in triplicate.

Determination of Yield and Encapsulation Efficiency of KAEN. The yield and encapsulation efficiency of a novel delivery system is one of the important indexes of successful pharmaceutical formulation. This method was conducted according to the method described previously by Wu et al.[12] In the determination of yield, 100 μL of KAEN solution was mixed with an equivalent aliquot of methanol and then diluted to an appropriate concentration for analysis using high performance liquid chromatography (HPLC). The encapsulation efficiency of KAEN used the centrifugal filter devices (Microcon YM-10, Millipore) centrifuged at 10 000 rpm for 30 min to separate the encapsulated and unencapsulated portions of KAE from the KAEN, and the amount of KAE was detected by HPLC. The HPLC system (Hitachi, Japan) consisted of a pump (L-7000), an autosampler (L-7200), a L-7420 UV−vis detector, and a D-7000 interface module. The calibration curve for KAE and the yield of KAEN solution were analyzed on a Lichro-CART 250-4 Purospher STAR RP-18e (250 × 4.6 mm i.d., 5 μm) column, and the temperature was kept constant at room temperature. The mobile phase was composed of 10 mM potassium dihydrogen phosphate buffer and acetonitrile (65:35) with the pH adjusted to 2.5 with hydrochloric acid. The flow rate was set at 0.8 mL min^{-1}, and the wavelength of detection was 325 nm. The resulting calibration curve of kaempferol was linear ($r = 0.9999$) within the range 1−100 μg/mL. The relative standard deviations of the intraday and interday analyses were less than 5% ($n = 3$). The yield and encapsulation efficiency of KAE could be calculated by following eqs 1 and 2:

$$\text{yield}\ (\%) = C_K \times (V_K/W_K) \times 100 \quad (1)$$

$$\text{encapsulation efficiency}\ (\%) = [(C_K \times V_K) - (F_K \times V_K)]/C_K V_K \times 100 \quad (2)$$

where C_K was the concentration of KAE from the KAEN, W_K was the theoretical amount of KAE added, V_K was the volume of KAE from the KAEN, and F_K was the concentration of KAE in the unencapsulated portion.

Dissolution Studies of KAE and KAEN. Dissolution studies of KAE and KAEN were performed in a pH 1.2 hydrochloric acid buffer solution (USP XXIX). Samples equivalent to 5 mg of KAE were placed in the 100 mL of buffer solution and stirred with a rotating paddle at 100 rpm. Subsequently, 1 mL of each sample was withdrawn after 1, 5,

Table 1. Mean Particle Size, Polydispersity, Yield, and Encapsulation Efficiency of the Kaempferol Nanoparticle System[a]

	particle size (nm)	polydispersity	yield (%)	encapsulation efficiency (%)
KAE	6207.9 ± 2686.9	1.53 ± 0.20		
KAEN	87.8 ± 1.67[b]	0.29 ± 0.02[b]	83.2 ± 5.76	>99.9

[a] All determinations were performed in triplicate, and values are expressed as mean ± SD, $n = 3$. [b] Significantly different from KAE ($P < 0.001$).

10, 20, 40, 60, 80, 100, and 120 min, and then filtered through a 0.45 μm filter (Millex-HV Millipore). The concentrations of KAE and KAEN at the various time points were determined by HPLC analysis.

Determination of Antioxidant Activities of KAE and KAEN. In the present study, we used several assays to validate the antioxidant activities of KAEN dissolved in distilled water and compared them to empty nanoparticles (BKNP), pure KAE dissolved in ethanol (KAE, positive control), and distilled water (KAED). The preparation of test samples was as follows: 1 mg of KAEN or BKNP was dissolved in 1 mL of distilled water and used as the stock solution of KAEN. Next, 1 mg of kaempferol was dissolved with 1 mL of 95% ethanol and used as the stock solution of KAE (positive control). In addition, 20 mg of kaempferol was added in 10 mL of distilled water and placed in a supersonic water bath for 10 min. After that, the suspension was centrifuged at 1000 rpm for 10 min, and the supernatant was used as stock solution of KAED. The stock solutions of all samples were diluted in a series of concentrations with distilled water. DPPH free radical scavenging activity was determined using the method of Yen et al.,[24] and the superoxide anion scavenging activity was estimated by the reduction of cytochrome *c* using the method described by McCord and Fridovich.[25] The data were expressed as the scavenging concentration 50 (SC_{50}), which denotes the concentration of each sample required to scavenge 50% of the free DPPH and superoxide anion radicals, respectively.

The reducing power assay was measured as described by Yen et al.[26] A standard curve of a serial dilution of ascorbic acid was used to calculate the equivalent reducing power of each sample. The inhibition of xanthine oxidase determined the formation of superoxide anion according to Chang et al, [27] and the ferric chloride/ascorbic acid-induced lipid peroxidation in rat liver homogenate was performed by the thiobarbituric acid method according to Ohkawa et al.[28] As for the data, inhibition concentration 50 (IC_{50}) values indicated the concentration of each sample that could inhibit the production of superoxide anion radicals and lipid peroxidation. All determinations were performed in triplicate.

Statistical Analysis. All data were expressed as means ± standard deviations of the indicated number of experiments. Data were analyzed by one-way analysis of variance (ANOVA) and a posthoc test of Scheffe's method test to calculate statistical significance using SPSS software. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Enhancing the dissolution percentage of poorly water-soluble compounds is a prerequisite for increasing the absorption of a drug to improve its pharmacological effect. Previous studies have also demonstrated that improvement of physicochemical characterization is important to significantly enhance the solubility and dissolution rate of hydrophobic compounds by reducing particle size, amorphous transformation, and hydrogen bonding with excipients.[29] In the present study, we successfully developed a kaempferol nanoparticle system using a simple nanoprecipitation technique, and the results suggest that the nanoparticle delivery system could improve the physicochemical properties of KAE and further elucidate its mechanism of enhanced dissolution. In addition, the antioxidant activity of KAE was found to be retained after the nanoparticle engineering process.

Reducing Particle Size of KAE by Nanoparticle Engineering Process. Noyes and Whitney first formulated an equation to relate the dissolution rate of compounds, and, over the past decade, this equation has been used to explain the dissolution rate of hydrophobic compounds in numerous studies.[30] The equation indicates that reducing particle size is a well-known and efficient method to enhance the dissolution percentage of hydrophobic compounds.

Ratnam et al. have reported that a nanoparticle engineering process was an effective strategy to resolve some of the drawbacks of hydrophobic compounds.[16,31] In our study, the mean particle size and PI of KAE and KAEN determined by photon correlation spectroscopy are shown in Table 1. The particle size and PI of KAE were 6207.9 ± 2686.9 nm and 1.53 ± 0.20, respectively. After the nanoparticle engineering processes, however, the particle size and PI value of KAEN were 87.8 ± 1.67 nm and 0.29 ± 0.02, respectively. In addition, we performed a check on the particle size of KAEN after 1 year. The particle size of KAEN was not obviously changed (87.8 ± 1.67 nm), and the PI value was slightly increased (0.41 ± 0.03), respectively. The result indicated that KAEN is a stable nanoparticle formulation to support its validity. PI is a dimensionless measure of the broadness of the particles size and lies between 0 and 1, which was obtained by PCS analysis. If the PI value is less than 0.3, this indicates the particle size of the sample is of narrow distribution. However, when the PI value is more than 1, this indicates that the particle size of the sample is of broad distribution. In addition, TEM photography indicated that KAEN had a smaller spherical shape and more uniform size distribution, as well as particle sizes less than 100 nm, when compared to KAE (Figure 2). These findings indicated that the presence of EE100 and PVA of KAEN not only had significantly reduced particle sizes when compared to KAE, but also had a more uniform particle size. This phenomenon is consistent with the reports of Karavas et al.[32] Therefore, KAEN might enhance the dissolution percentage of KAE by reducing the particle size of KAE using this nanoparticle engineering process.

The Crystal-to-Amorphous Transformation of KAE during the Nanoparticle Engineering Process. Blagden et al. have reported that crystal engineering processes are an alternative approach available for the enhancement of solubility and dissolution of active pharmaceutical ingredients.[33] Also of importance, the transformation of highly energetic amorphous materials has been applied to resolve the solubility and dissolution rates of hydrophobic compounds using other preparation techniques, such as solid dispersion[16] and nanoparticle engineering.[33]

DSC and XRD have been previously effective at elucidating the crystal-to-amorphous transformations between excipients and hydrophobic compounds and were therefore applied in this study. In our preliminary study, we used a lower ratio of KAE:EE:PVA (1:1:1; w/w/w) to prepare KAEPM and KAEN. Unfortunately, a lower ratio of EE and PVA did not successfully prepare KAEN (particle size >1000 nm and PI > 1), and its endothermic peaks of a lower ratio KAEPM and KAEN were also absent (data not shown). The finding indicated that KAE could be fused with the EE and PVA during the heating procedure, and a similar observation has been reported by Tantishaiyakul et al.[34] Interestingly, the XRD data showed that a low ratio KAEPM had a series of crystal peaks, but these peaks were not found in low ratio

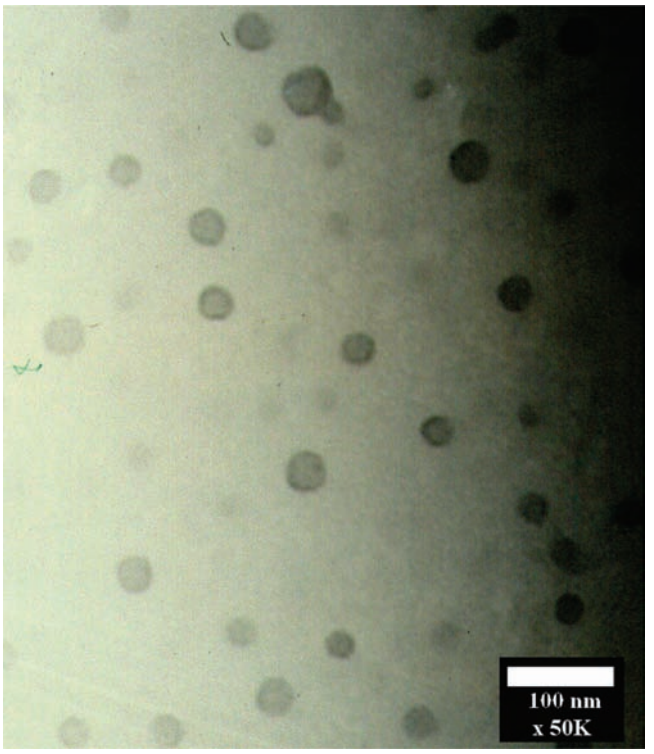

Figure 2. The TEM photograph of the kaempferol nanoparticle systems.

KAEN. We suggested that nanoprecipitation could be formed a high-energy amorphous state with exicpients. Figure 3A shows the DSC thermograms of KAE, KAEN, their physical mixture (KAEPM), and a blank excipient (BKPM). A sharp endothermic melting peak for KAE was obtained at 285.5 °C, clearly indicating its crystalline nature. However, the endothermic melting peak of KAE was not observed for KAEN or KAEPM. In addition, Figure 3B shows the change of crystalline structure in KAE by X-ray diffraction patterns. KAE had a highly crystalline structure and a small crystalline peak, indicating this was still present for KAEPM. After the nanoparticle engineering, however, the KAEN had an amorphous pattern, and the crystalline peaks seen in KAE disappeared. According to the results of DSC and XRD, KAE was dispersed into the carriers, and the clustered crystalline structure was transformed into an amorphous structure by intermolecular interactions with the matrix. This observation was because EE100 is an amorphous material that suppresses the recrystallization of KAE by forming a high-energy amorphous state during the nanoparticle engineering process or in the physical mixture. Jung et al. also reported that EE100 had the same effect in solid dispersions.[35]

Intermolecular Interactions between KAE and EE100-PVA That Increased the Dissolution. Previous studies have demonstrated that intermolecular interaction between active pharmacological compounds and excipient effectively improved dissolution in different preparations, such as solid dispersions[16] and nanoparticle engineering processes.[33] The FT-IR spectra of KAE, KAEN, its physical mixture (KAEPM), and a blank excipient (BKPM) are shown in Figure 3C. The KAE spectrum shows the characteristic absorption band of C═O at 1662 cm^{-1}, and the peak at 3322 cm^{-1} indicates the OH stretch of KAE. The physical mixture (KAEPM), by comparison, showed a C═O absorption

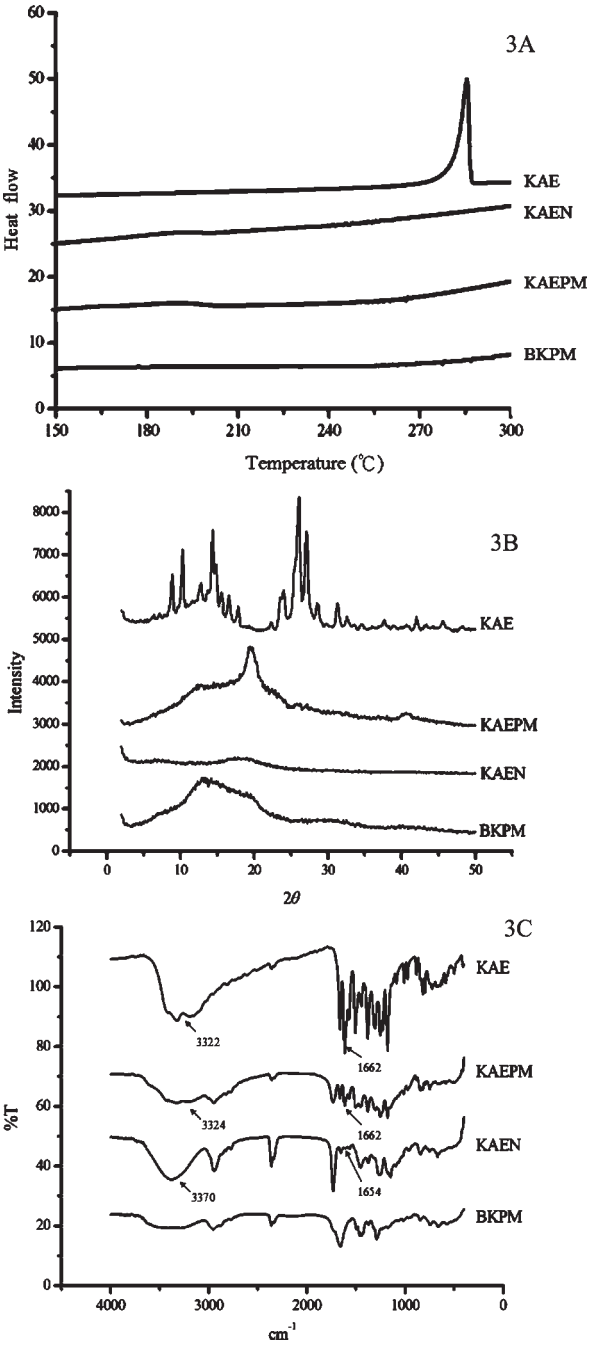

Figure 3. DSC (A), XRD (B), and FT-IR (C) patterns of KAE, KAEN, its physical mixture (KAEPM), and a blank excipient (BKPM).

band at 1662 cm^{-1} and an OH stretch at 3324 cm^{-1}. Furthermore, the KAEN spectrum showed that the C═O absorption curve of KAE shifted to the lower wavenumber of 1654 cm^{-1} and the OH stretch of KAE completely disappeared at 3370 cm^{-1}. The result indicated that the intramolecular hydrogen bonding was formed between KAE and EE100-PVA after nanoparticle engineering process.

As Figure 4 displays, the ^{1}H NMR spectrum confirmed that KAE had a clear signal for strong intramolecular hydrogen bonds at 12.48 ppm, whereas a broad signal was evident at the same position in the ^{1}H NMR spectrum of KAEN. The NMR data also indicated that the aromatic protons (H6 and H8) of KAE were

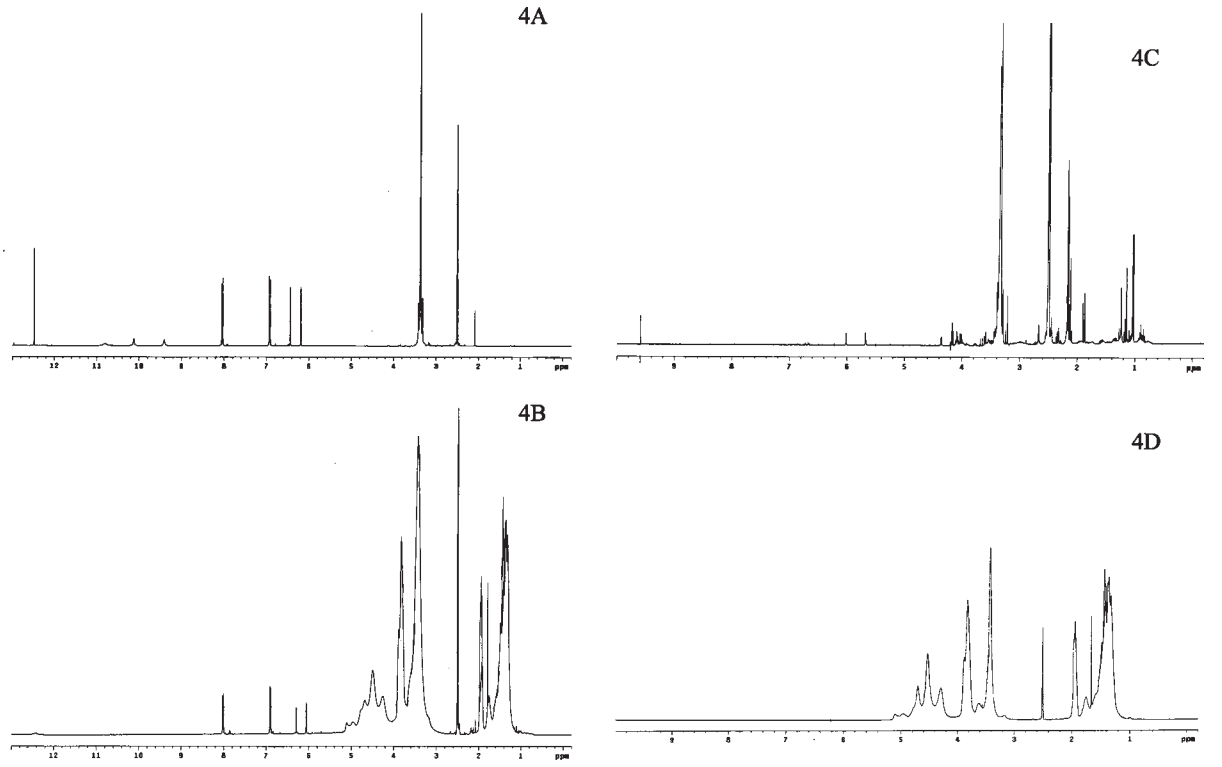

Figure 4. ^{1}H NMR spectra of KAE (A), KAEN (B), EE100 (C), and PVA (D).

clearly shifted by about 0.1 ppm after the nanoparticle engineering process. These data demonstrated that the A-ring of KAE effectively formed an intermolecular hydrogen bond with the EE100. Additionally, Yamamura et al. have been reported that liquid-state ^{1}H NMR was used to demonstrate the formation of intermolecular hydrogen bonding between cimetidine and indomethacin.[36] Horisawa et al. also demonstrated the tertiary amine of EE100 forming an intermolecular hydrogen bond with an anti-inflammatory drug.[37] Furthermore, PVA is usually used as an emulsion stabilizer to form stable nanoparticle systems.[38] The hydrophilic and hydrophobic portions of PVA would be beneficial for penetrating into the KAE−EE complex during the nanoprecipitation process. As such, Sonaje et al. demonstrated that PVA is a good stabilizer for nanoparticle systems.[39]

The Correlation between Encapsulation Efficiency and Dissolution. Previous reports have demonstrated that reduction of particle size, amorphous transformations, and intermolecular hydrogen-bond formation all effectively increase the encapsulation efficiency of drugs and further enhance their drug release.[12,13] Encapsulation efficiency is a factor to evaluate drug release from pharmaceutical formulations, such as liposomes,[40] microspheres,[41] and nanoparticles.[42] Additionally, Mora-Huertas et al. have reported that the method of nanoparticle preparation and the choice of polymer were the major factors affecting the success of nanoparticle formulations on the drug release.[43]

In the present study, we found that modified nanoprecipation was the best method for the preparation of KAEN. We also tried several polymers in the preparation of the best formulation of KAEN, including EE100, PVA, PVP, and cyclodextrin. Finally, EE100−PVA was found to be the best copolymer for KAEN, with the different proportion of copolymer used being the decisive factor in achieving successful KAEN preparations (data not shown). The KAEN system (KAE:EE:PVA at 1:10:10 w/w/w) had an encapsulation efficiency greater than 99%, and its yield was 83.2 ± 5.76% (Table 1). On the basis of the above results, KAE interacted with EE100 through hydrogen bonding, and PVA helped to stabilize the nanoparticle system, both of which effectively increased the encapsulation efficiency of the nanoparticle engineering process.

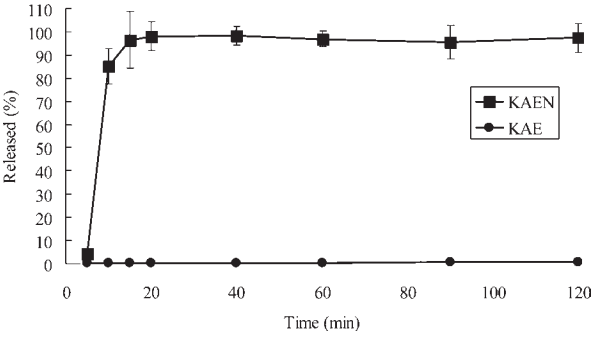

Figure 5. Dissolution profiles of kaempferol (KAE, ●) and its nanoparticle system (KAEN, ■). Values are expressed as mean ± SD, $n = 6$.

Many pure compounds have been developed into many new pharmaceutical formulations due to their poor water solubility and dissolution, such as spironolactone,[44] nitrendipine,[45] and oleanolic acid.[46] In our present study, Figure 5 compares the dissolution profiles of pure KAE and KAEN in pH 1.2 buffer solutions. The dissolution percentage of pure KAE was less than 1% at all experimental time points. In comparison, it was evident that the nanoparticle engineering process enhanced the dissolution percentage of KAE, as more than 95% of KAE was dissolved in the first 15 min. Furthermore, the dissolution percentage of the drug from the KAEN was increased 139-fold as compared to that

Table 2. Antioxidant Activities of KAE, KAEN, KAED, and BKPM[a]

	KAED[b]	KAE[c]	KAEN[d]	BKNP[e]
DPPH SC_{50} (μg/mL)	18 745.74 ± 3032.45	22.08 ± 3.71[g]	14.71 ± 2.16[g]	NA[f]
reducing power (equivalent to ascorbic acid, μg/mL)	2.43 ± 1.19	106.50 ± 4.36[g]	122.59 ± 2.02[g]	NA[f]
superoxide SC_{50} (μg/mL)	904.57 ± 57.80	1.25 ± 0.06[g]	10.03 ± 0.32[g]	NA[f]
antisuperoxide formation IC_{50} (μg/mL)	263.39 ± 29.90	4.13 ± 0.79[g]	2.30 ± 0.40[g]	NA[f]
antilipid peroxidation IC_{50} (μg/mL)	811.05 ± 31.87	14.70 ± 1.49[g]	14.47 ± 0.16[g]	NA[f]

[a] All determinations were performed in triplicate, and values are expressed as mean ± SD, $n = 3$. [b] KAED: KAE dissolved in distilled water. [c] KAE: KAE dissolved in ethanol (positive control). [d] KAEN: KAEN dissolved in distilled water. [e] BKNP: Empty nanoparticles dissolved in distilled water. [f] NA: No activity. [g] Significantly different from KAED ($P < 0.05$).

of the pure KAE. These findings may indicate that pure KAE could be released from the nanoparticle system by the gastro-soluble EE100 polymer into the stomach because of the highly acidic environment. The released nanoparticulate form of the pure KAE may then offer pharmacological activities from KAEN in vitro or in vivo.

Validation of KAEN in Antioxidant Activity. KAE acts as a good antioxidant and prevents oxidative damage in the body. However, several factors influence the pharmacological effects of KAE and limit its application, especially pharmaceutical preparations. In our present study, we used several assays to validate the antioxidant activities of KAEN and BKNP dissolved in distilled water and compared them to pure KAE dissolved in ethanol (KAE, positive control) or distilled water (KAED). The data for antioxidant activity are shown in Table 2.

In the DPPH free radical scavenging activity assay, the SC_{50} value (concentration of sample required to scavenge 50% of DPPH free radicals) was used to compared the free radical scavenging effect of different samples. The SC_{50} of KAE (22.08 ± 3.71 μg/mL) showed an excellent effect. The SC_{50} of KAEN (14.71 ± 2.16 μg/mL) was approximately the same as that of KAE, but 1247-fold stronger than that of KAED (18 745.74 ± 3032.45 μg/mL) ($P < 0.05$). In addition, 250 μg/mL KAEN and KAE were equivalent to 122.59 ± 2.02 and 106.50 ± 4.36 μg/mL of ascorbic acid, respectively. The result showed that KAEN and KAE had similar reducing power. However, the reducing power of KAED (equivalent to 2.43 ± 1.19 μg/mL ascorbic acid) showed a significantly weaker reducing activity (50.6-fold) when compared to KAEN ($P < 0.05$). According to these results, the free radical scavenging effect of KAE was attributed to the fact that the phenolic hydroxyl group of the KAE molecule could donate a hydrogen to stabilize the free radical and further prevent oxidative damage.

The next assay took advantage of the transformation of ferricytochrome *c* to ferro-cytochrome *c* through the conversion of superoxide anion to oxygen in the cytochrome *c*-xanthine oxidase system, and the xanthine oxidase conversion of hypoxanthine to xanthine to produce uric acid as a terminal metabolite within the xanthine−xanthine oxidase system. However, xanthine oxidase activation participated in many oxidative stress and inflammation conditions, such as ischemia reperfusion and diabetes. Our data showed that the SC_{50} of KAE (1.25 ± 0.06 μg/mL) and KAEN (10.03 ± 0.32 μg/mL) indicated good superoxide anion scavenging effects and that KAEN showed a 90.7-fold stronger superoxide anion-scavenging activity than did KAED (904.57 ± 57.80 μg/mL) ($P < 0.05$). The 50% inhibition concentration for antisuperoxide formation (IC_{50}) of KAEN (2.30 ± 0.40 μg/mL) was better than that of KAE (4.13 ± 0.79 μg/mL). In addition, KAEN showed a significantly stronger antisuperoxide formation activity than did KAED (263.39 ± 29.90 μg/mL) ($P < 0.05$).

Overproduction of free radicals can cause destruction by lipid peroxidation in the cell membrane and lead to cell inflammation, damage, and death. As Table 2 shows, KAE (IC_{50} = 14.70 ± 1.49 μg/mL) and KAEN (IC_{50} =14.47 ± 0.16 μg/mL) had similar antilipid peroxidation activity in ferrous chloride-vitamin C-induced lipid peroxidation in rat liver homogenate. In contrast, the KAED (IC_{50} = 811.05 ± 31.87 μg/mL) was observed to exhibit only weak activity (56-fold less) when compared to KAEN and KAE ($P < 0.05$).

As mentioned above, KAE is a good antioxidant, but it is also easily oxidized by light and heat. The high-speed homogenization used in this study easily produced heat, and evaporation of ethanol occurred in the light during the nanoprecipitation method. These factors will therefore influence the antioxidant activity of KAE. However, the antioxidant activity data demonstrated that KAEN retained free radical scavenging activity, reducing power, superoxide anion scavenging activity, and antixanthine oxidase and antilipid peroxidation activity after the nanoparticle engineering process.

In conclusion, the present study demonstrated that the kaempferol nanoparticle system (KAEN) was successfully synthesized using a modified nanoprecipitation technique. Our studies also established that the release mechanism of KAE from the KAEN was attributed to the reduction in drug particle size, the formation of a high-energy amorphous state, and intermolecular hydrogen bonding. Additionally, the increases in antioxidant activities of KAE from the KAEN were correlated with improvements in physicochemical and dissolution properties. Thus, we suggest that KAEN may be usefully applied in clinical settings and warrants further study.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +886-7-3121101 ext. 2122. Fax: +886-7-3135215. E-mail: aalin@kmu.edu.tw (C.-C.L.); tzengg@ms31.hinet.net (W.-S.T.).

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