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Research Article

Increased *In Situ* Intestinal Absorption of Phytoestrogenic Diarylheptanoids from *Curcuma comosa* in Nanoemulsions

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Abstract. *Curcuma comosa* has long been used as a gynecological medicine. Several diarylheptanoids have been purified from this plant, and their pharmacological effects were proven. However, there is no information about the absorption of *C. comosa* components to support the formulation usage. In the present study, *C. comosa* hexane extract and the mixture of its two major compounds, (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-ol (DA1) and (6*E*)-1,7-diphenylhept-6-en-3-ol (DA2), were formulated into nanoemulsions. The physical properties of the nanoemulsions and the *in situ* intestinal absorptions of DA1 and DA2 were evaluated. The results demonstrated the mean particle sizes at 0.207±0.001 and 0.408 ±0.014 μm, and the zeta potential at −14.57±0.85 and −10.47±0.32 mV for *C. comosa* nanoemulsion (C.c-Nano) and mixture of diarlylheptanoid nanoemulsions (DA-Nano), respectively. The entrapments of DA1 and DA2 were 76.61% and 75.41%, and 71.91% and 71.63% for C.c-Nano and DA-Nano, respectively. The drug loading ratios of DA1 and DA2 were 351.47 and 614.53 μg/mg, and 59.48 and 126.72 μg/mg for C.c-Nano and DA-Nano. The intestinal absorption rates of DA1 and DA2 were 0.329±0.015 and 0.519±0.026 μg/min/cm^2 in C.c-Nano, and 0.380±0.006 and 0.428±0.036 μg/min/cm^2 in DA-Nano, which were five to ten times faster than those in oil. In conclusion, the formulation in nanoemulsion forms obviously increased the intestinal absorption rate of diarylheptanoids.

KEY WORDS: *Curcuma comosa*; diarylheptanoids; intestinal absorption; nanoemulsion; phytoestrogen.

INTRODUCTION

The medicinal plant *Curcuma comosa* Roxb. (Zingiberaceae) has been widely used in Southeast Asia for the treatment of uterine inflammation, postpartum uterine bleeding, premenopausal bleeding, and as an aromatic stomachic. The pharmacological effects of its hexane extract have been reported including its estrogenic-like functions (1–3), anti-inflammatory effects (4), choleretic activities (5,6), hypolipidemic effects (7,8), and memory improvement (9,10). Among many traditional herbs in Thailand, the formulations of herbal medicines containing *C. comosa* are popular in the market for gynecological conditions. However, all clinical reports related to *C. comosa* are empirical, and no information on the absorption from different formulations has been reported. Recently, several diarylheptanoids had been purified from *C. comosa* extract, and their pharmacological effects were reported. Two specific molecules, (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-ol (DA1) and (6*E*)-1,7-diphenylhept-6-en-3-ol (DA2) (Fig. 1), were reported as the major compounds, which comprised 16.02% and 30.66% of the crude extract, respectively (11). The estrogenic effect of these two diarylheptanoids was demonstrated in cell culture studies; thus, these compounds were considered as potential phytoestrogens (12).

The pharmacokinetic profiles of DA1 and DA2 were also studied in animals (11). The *C. comosa* extract that had been diluted in olive oil was shown to have bioavailability of 24.01% and 34.56% for DA1 and DA2, respectively, after oral administration. Although increasing the administrated dose resulted in a greater maximum blood concentration, lower bioavailability was observed most likely due to the slower absorption process. The *C. comosa* hexane extract and the two diarylheptanoids are lipophilic. Using XLOGP3 software (13) calculation, the log *P* values of DA1 and DA2 are 4.36 and 4.87, respectively. The poor water solubility of these diarylheptanoids was of concern when considering an appropriate formulation for sufficient systemic delivery of these active phytoestrogens.

Nanotechnology has been widely used in the development of formulations of poorly soluble compounds. Nanoemulsions usually have droplet sizes at the scale of nanometers, and these can provide increased apparent water solubility, thermal stability, and bioavailability, consequently resulting in improved pharmacological effects. The benefit of nanoemulsions has been demonstrated with herbal products such as curcumin (14,15), St. John's wort extract (16), and *Kaempferia parviflora* extract (17). Moreover, nanoemulsions offer the potential to significantly improve the stability and bioavailability of certain carotenoids such as beta-carotene and astaxanthin (18–20). The main purpose of the present study was to determine an improvement in

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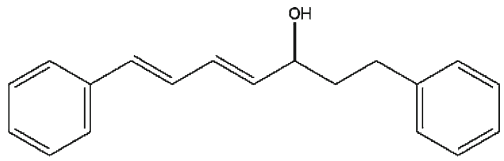
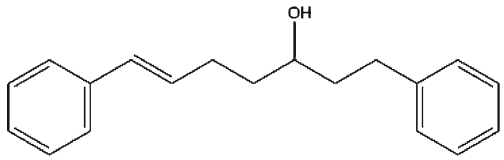


(4*E*,6*E*)-1,7,-diphenylhepta-4,6-dien-3-ol (DA1)



(6*E*)-1,7,-diphenylhept-6-en-3-ol (DA2)

Fig. 1. Structures of the two diarylheptanoids (DA1 and DA2)

the intestinal absorption of *C. comosa* and the diarylheptanoids when formulated as a nanoemulsion. The related physical characteristics of *C. comosa* extract and diarylheptanoids nanoemulsions were studied, and the *in situ* intestinal absorption of the formulations was evaluated in a commonly used animal model (21,22). This is a preliminary study to test for the possibility to use *in situ* intestinal absorption in rats for measuring the absorption of active compounds of the *C. comosa* crude extract. The result provides simultaneous absorption status of the mixture of active compounds in *C. comosa*.

MATERIALS AND METHODS

Chemicals and Animals

Polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene glycol 1000 (cetomacrogol 1000), and sodium chloride were obtained from Asia Pacific Specialty Chemicals Limited (Australia). Hexane and dichloromethane (HPLC grade) were purchased from Fisher Scientific (UK). Olive oil (Sabroso®, Spain) was obtained from a local vendor. Phenol red was obtained from Sigma-Aldrich Chemicals (USA).

Female Wistar rats (8 weeks) were obtained from the National Animal Center, Mahidol University, Bangkok. The rats were housed in a room with a controlled temperature of 25±2°C and 12-h dark/light cycles. Standard food (C.P. Ltd.; code, 082) and water were supplied *ad libitum*. The experiments were conducted under the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) revised in 1996 and approved by the Ethics Committee on Animal Care and Use of Khon Kaen University.

Plant Extract and the Purified Compounds

C. comosa rhizome was harvested from Nakon Pathom province, Thailand (identified and provided by Professor Pawinee Piyachaturawat at Mahidol University, Thailand). A voucher specimen was filed and kept in our laboratory (BS-C-03). *C. comosa* hexane extract was prepared before the absorption test following our previous report (9). Briefly, dried samples of *C. comosa* were mashed to a crude powder and subsequently extracted in *n*-hexane using a Soxhlet apparatus until the outlet hexane was colorless. The hexane fraction was then evaporated to obtain a brown–yellow oily extract which was kept at 4°C until use. DA1 and DA2 (>98% purified) were kindly provided by Professor Apichart Suksamrarn at Ramkhamhaeng University, Thailand. DA1 and DA2 were used to standardize the crude extract, in which the concentration of DA1 and DA2 were 153 and 313 mg/g, respectively. A HPLC test was run to make sure the extract was stable during the storage.

HPLC Method Validation and Analysis

An Agilent LC 1200 HPLC system was used with mobile phase consisting of 35% of *n*-hexane and 65% of dichloromethane. A Hypersil silica column (250 mm×4.6 mm, 5 μm, Thermo®) was used, and the column temperature was set at 25°C. The flow rate was maintained at 1.5 mL/min, and the detected wavelengths were 302 nm for DA1 and 250 nm for DA2. To prepare the standard references, these two purified diarylheptanoids were separately dissolved in mobile phase to prepare a five-gradient concentration of 0.25, 0.05, 0.01, 0.002, and 0.0004 mg/mL followed by the HPLC analysis. Correlation coefficients (r^2) were subsequently obtained by linear regression analysis. Inter-day and intra-day precisions were analyzed; the peak areas of DA1 and DA2 were used for calculating the coefficient of variation (percent CV). Accuracy and recovery of the DA1 and DA2 in different solutions were also evaluated.

For HPLC analysis, the sample was extracted by hexane *via* vigorous shaking and ultrasonication for 10 min, followed by a 10-min centrifugation at 10,000×*g*. The upper liquid layer in the centrifuge tube was transferred to an evaporator dish and dried at room temperature. This extraction process was repeated in triplicate. The residue in the evaporator dish was redissolved in 500 μL of mobile phase for HPLC analysis.

Preparation of Nanoemulations

The oil-in-water nanoemulsion was prepared in two steps using a co-surfactant method. The oil phase consisted of two nonionic surfactants (polyoxyethylene sorbitan monostearate or Tween 60, and polyoxyethylene glycol 1000 or cetomacrogol 1000) and olive oil (Table I). *C. comosa* extract or the mixture of DA1 and DA2 were separately added to the warm oil phase at 70°C and mixed for 10 min using a magnetic stirrer. The water phase (75°C) was then added to form the premix. After homogenization using a homomixer (CKL, Selangor, Malaysia) at a speed of 2,000 rpm for 20 min, the pre-emulsion was passed through a high-pressure homogenizer (M-110P, Microfluidics Corporation, Newton, MA, USA) at 10,000 psi for five cycles to yield the nanoemulsions. The final concentration of *C. comosa* extract nanoemulsion (C.c-Nano) was 0.83 mg/mL, which contained DA1 and DA2 at concentrations of 0.13 and 0.26 mg/mL, respectively. The same concentrations of DA1 and DA2 were prepared in the diarylheptanoid mixture nanoemulsion (DA-Nano).

Preparation of *C. comosa* Extract and Mixture of Diarylheptanoid in Olive Oil

The *C. comosa* extract was diluted in olive oil to a concentration of 0.83 mg/mL to produce the *C. comosa*–oil solution (C.c-oil). A mixture of the diarylheptanoids was prepared by dissolving DA1 and DA2 in olive oil to obtain the solution DA-oil containing 0.13 and 0.26 mg/mL of DA1 and DA2, respectively. All of these four formulations were tested by HPLC to make sure they were stable at 37°C for 24 h.

Particle Size and Zeta Potential

The particle sizes of nanoemulsions were determined by photon correlation spectroscopy using a Mastersizer (version 5.22, Malvern® Instruments, UK). The zeta potentials of the nanoemulsions were measured using a Zetasizer (Nano Z, Malvern® Instruments, UK).

Morphology of the Nanoemulsions

The morphologies of the C.c-Nano and DA-Nano nanoemulsions were obtained by using a scanning electron microscope (Hitachi® model S-3000N, Tokyo, Japan). Briefly, a drop of a freshly prepared formulation was directly applied on the holey film grid followed by drying at room temperature overnight. The sample was coated with gold for 20 min using an ion sputtering device before the scanning.

Entrapment Efficiency and Drug Loading Ratio of Nanoemulsions

Entrapment efficiency and drug loading ratio of the nanoemulsions were determined using a centrifugal filtration device (Microcon® Milllipore, Billerica, MA, USA) fitted with a 100-kDa molecular weight cutoff filter. Two hundred microliters of the nanoemulsion was added to the sample reservoir of the Microcon® system and then centrifuged at 1,500×*g* for 45 min at 4°C to separate the entrapped and unentrapped contents. The sample reservoir was washed twice with 100 μL of deionized water and was followed by collection of the entire filtrate. The diarylheptanoids entrapped on the filter as well as the unentrapped drug in the filtrate were analyzed by HPLC. The entrapment efficiency (percent EE) and drug loading ratio (DL ratio) were calculated using the following equations:

$$\%\mathrm{EE} = \frac{\text{entrapped diarylheptanoids (mg)}}{\text{entrapped diarylheptanoids} + \text{unentrapped diarylheptanoids(mg)}} \times 100$$

$$\text{DL ratio} = \frac{\text{entrapped diarylheptanoids}(\mu\text{g})}{\text{olive oil in formulations (mg)}}$$

In Situ Absorption Study

All animals were fasted 24 h before the initiation of the experiments and were allowed water *ad libitum*. Twelve animals were randomly separated into four groups (n=3) and administered the four formulations, including C.c-Nano, DA-Nano, C.c-oil, and DA-oil. The animals were anesthetized under ether and then positioned on the operation stage in supine position. Ether was carefully supplied by observing the respiration of the animals to make sure of the anesthetic status. A 5-cm incision was made in the abdomen to expose the intestines. Two incisions were made in the small intestine; a sterile influent tube was inserted in the proximal end, and an effluent tube was connected to the distal end, both of which were ligated to prevent leakage. After washing out the intestinal contents with normal saline at 37°C, the drug-containing sample at 37°C from a sample pool was pumped through the intestine at a flow rate of 0.4 mL/min, and the effluent was recycled into the sample pool. After achieving a constant flow rate, 200 μL of solution was collected from the sample pool at every 10 min for 1 h. The effluent volume of each collected sample was measured and used for compensating the volume absorbed by the intestine. After all samples had been collected, the animals were sacrificed by ether overdose. The surface area of the small intestine segment used in the perfusion study was measured. The final absorption rates of the two diarylheptanoids were expressed as microgram per minute per square centimeter. All perfusion studies in rats were performed in triplicate for each formulation. The blank nanoemulsion (without the plant extract) containing a nonabsorbable marker, phenol red, at a

Table I. Formulations of *C. comosa* Extract Nanoemulsion (C.c-Nano) and Mixture of Diarylheptanoid Nanoemulsion (DA-Nano)

	Composition (g)	C.c-Nano	DA-Nano
Oil phase	*C. comosa* extract	0.083	–
	DA1	–	0.013
	DA2	–	0.026
	Tween 60	0.083	0.250
	Cetomacrogol 1000	0.250	0.833
	Olive oil	0.250	0.833
Aqueous phase	Distilled water	99.334	98.045

DA1 (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-ol, *DA2* (6*E*)-1,7-diphenylhept-6-en-3-ol

concentration of 30 μg/mL was also tested as the control group (22).

RESULTS

HPLC Validation

This HPLC assay was able to achieve a good separation of DA1 and DA2 with retention times of 16.35 and 13.29 min when detected at the wavelength of 302 and 250 nm, respectively (Figs. 2c–f and 3c–f). The correlation coefficients of all calibration curves of these two compounds showed good linearity (r^2>0.999) over the test range. The percent coefficient of variation (%CV) of the method precision and accuracy were less than 0.75 and 0.77, respectively; these values were 1.39 and 1.13 for intra- and inter-day assays, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) of both DA1 and DA2 were 0.0001 and 0.0003 mg/mL, respectively. The average recovery was 96.8% for DA1 and 98.7% for DA2 (Table II). The after-perfused blank oil and nanoemulsion did not interfere with the detection of DA1 and DA2 under the current conditions (Figs. 2a, b and 3a, b). The results indicated that the analytical method was reliable and reproducible.

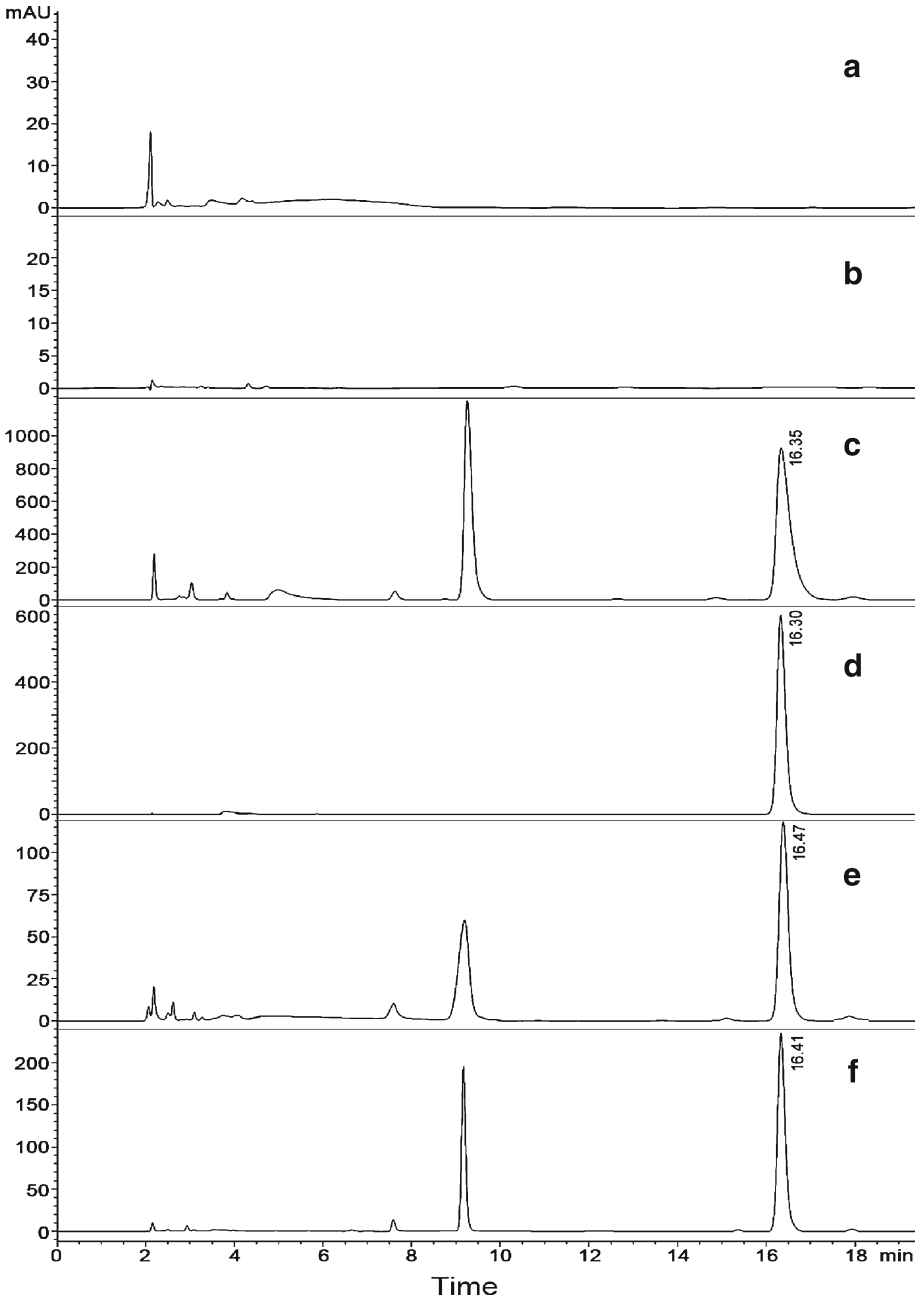


Fig. 2. HPLC chromatograms of DA1 in C.c-oil and C.c-Nano detected at 302 nm. **a** After-perfused blank oil solution, **b** after-perfused blank nanoemulsion, **c** *C. comosa* extract, **d** standard DA1, **e** C.c-oil, and **f** C.c-Nano

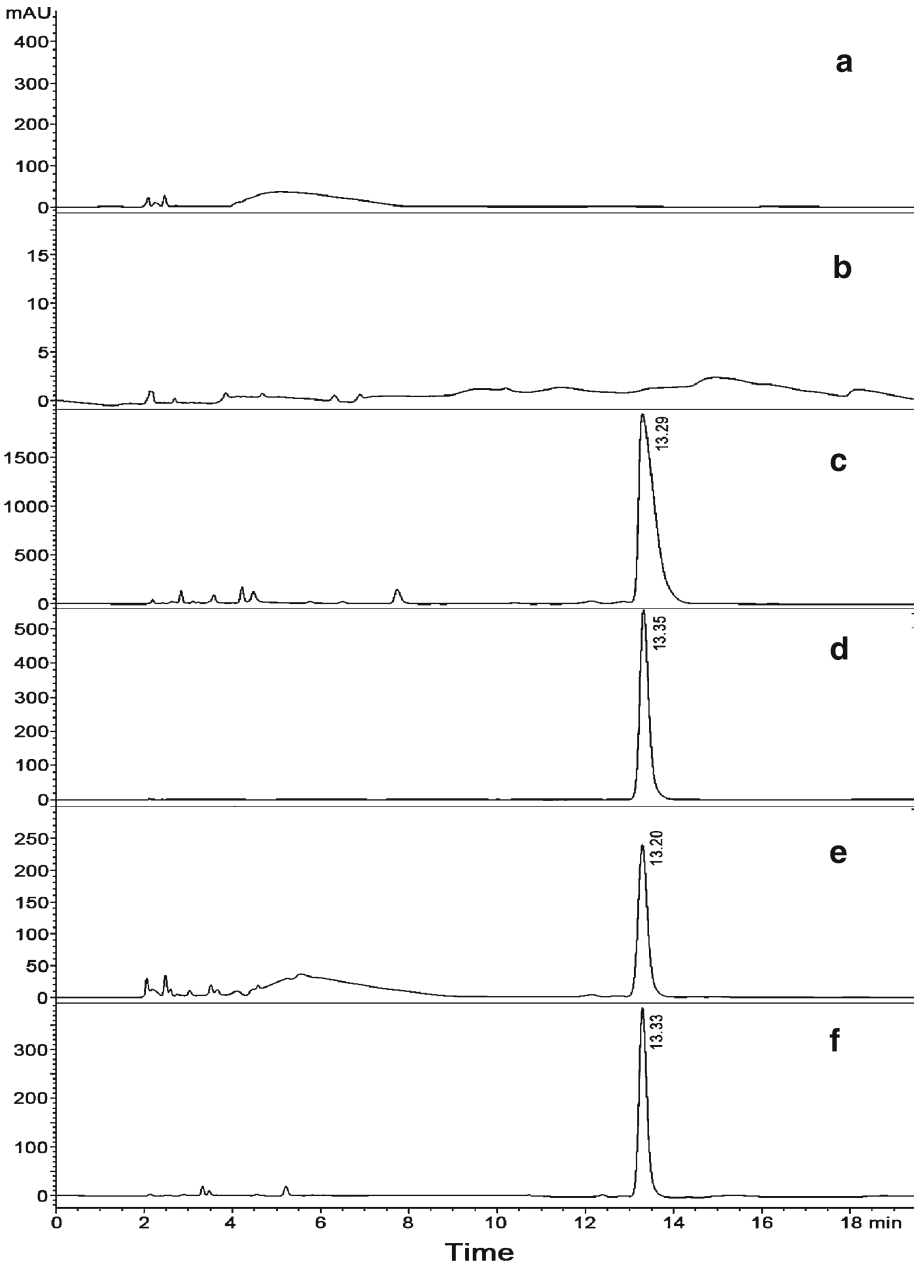


Fig. 3. HPLC chromatograms of DA2 in C.c-oil and C.c-Nano detected at 250 nm. **a** After-perfused blank oil solution, **b** after-perfused blank nanoemulsion, **c** *C. comosa* extract, **d** standard DA2, **e** C.c-oil, and **d** C.c-Nano

Table II. HPLC Method Validation of Standard Diarylheptanoids

Standards	Linear regression equation	r^2	LOD (mg/mL)	LOQ (mg/mL)	Precision (%CV)		Accuracy (%CV)		Recovery (%)
					Inter-assay	Intra-assay	Inter-assay	Intra-assay	
DA1	$y=79380x+270$	0.999	0.0001	0.0003	0.38	0.24	1.11	0.62	98.8
DA2	$y=79958x+236$	0.999	0.0001	0.0003	1.39	0.75	1.13	0.77	98.7

LOD limit of detection, *LOQ* limit of quantification, *%CV* percent coefficient of variation

Table III. Particle Size and Particle Size Distribution of the Nanoemulsions

Formulation	Particle size (μm)		
	d (0.1)	*d* (0.5)	*d* (0.9)
C.c-Nano	0.146±0.000	0.207±0.001	0.335±0.001
DA-Nano	0.296±0.001	0.408±0.014	0.483±1.449

C.c-Nano C. comosa nanoemulsion, *DA-Nano* mixture of diarylheptanoid nanoemulsions

Physical Characteristics of the Nanoemulsions

The particle size distributions of the nanoemulsions were uniform with mean values of 0.207±0.001 and 0.408±0.014 μm for the C.c-Nano and DA-Nano formulations, respectively (Table III, Fig. 4). When stored at 4°C, these two formulations were stable as it was observed that their mean particle sizes were not changed (0.208±0.001 and 0.414±0.002 μm, respectively). The entrapment efficiencies and drug loading ratios of DA1 and DA2 in the C.c-Nano and DA-Nano emulsions as well as the zeta potential of the nanoemulsions are reported in Table IV. These results showed that the entrapment efficiencies for DA1 and DA2 were similar for both nanoemulsions, but that the drug loading of these compounds was greater for the C.c-Nano emulsion. Both emulsions exhibited slightly negative zeta potentials.

In Situ Intestinal Absorption

After *in situ* intestinal perfusion of the blank nanoemulsion containing phenol red marker, the obtained perfusate showed no leakage of phenol red as it was observed that the concentrations of phenol red were not changed from the original blank nanoemulsion. The intestinal absorption rates of DA1 and DA2 from different formulations as shown in Table V demonstrated that the absorption of DA1 and DA2 in the nanoemulsions was more rapid than when they were formulated in oils. The absorption of DA2 when administered in the C.c-Nano emulsion was notably faster than when formulated in the DA-Nano emulsion. Compared to the formulation in oils, the concentrations of DA1 and DA2 in the sample pool of the formulation in nanoemulsions decreased more rapidly (Fig. 5). This is another indication that DA1 and DA2 were more rapidly absorbed when formulated in nanoemulsions.

DISCUSSION

Many *in vitro* studies have been conducted on the pharmacological activity of compounds that have been isolated and purified from plants. However, further development of most of these compounds has been aborted because effective blood concentrations could not be attained, primarily due to their physical–chemical properties such as solubility, partition coefficient, lipophilicity, and crystallinity. In the present study, the hexane extract of *C. comosa* and the two diarylheptanoids were lipophilic. In previous studies, the hexane extract of *C. comosa* extract was diluted in corn oil or olive oil to prepare formulations for administration of the animals (3,9). To reach a pharmacologically effective blood level, doses of 125–500 mg/kg body weight were administered (3,10). Pharmacokinetic analysis showed that the bioavailability of the major compounds (DA1 and DA2) of *C. comosa* extract were reduced by 35% when dosed at 125 mg/kg in olive oil and that this value was further reduced when the dose was increased to 250 mg/kg (11). Formulating the *C. comosa* extract and the diarylheptanoids into a nanoemulsion can improve the intestinal absorption and increase the absorption rates of DA1 and DA2 by five- to tenfold. Higher bioavailability with lower administered doses of *C. comosa* extract and diarylheptanoids was expected by formulating them in nanoemulsions. The improvement in the absorption of the nanoemulsion was evidently due to the smaller droplet sizes and the presence of surfactants which might aid access of the lipophilic compounds to the intestine mucosa. The improvement in absorption of the active compounds in the nanoemulsion was similar to that observed for other compounds such as St. John's wort extract in which formulation in a nanoemulsion resulted in a 2.8-fold increase in bioavailability compared to the general extract formulation (16). Moreover, a curcumin nanoemulsion was reported to be comparable to the activity of free curcumin *in vitro* against a panel of human pancreatic cancer cell lines (14).

In this study, the nanoemulsions were prepared by a two-step homogenization method. The first step was performed to yield a pre-emulsion; this was followed by high-pressure homogenization to generate a nanosized diarylheptanoid emulsion. Both Tween 60 and cetomacrogol 1000 are nonionic surfactants that have been widely used in the pharmaceutical products based on their safety and compatibility. The amounts

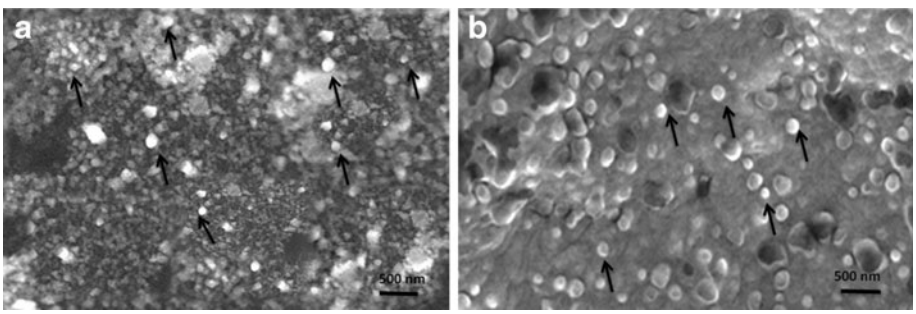


Fig. 4. Electron microscope scanning showing the morphology of C.c-Nano **a** and DA-Nano **b**

Table IV. Percentage Entrapment Efficiency (%EE), Drug Loading Ratio, and Zeta Potential of the Nanoemulsions

	%EE		Drug loading ratio (μg/mg)		
Formulation	DA1	DA2	DA1	DA2	Zeta potential (mV)
C.c-Nano	76.61±2.31	75.41±1.65	351.47±23.76	614.53±43.18	−14.57±0.85
DA-Nano	71.91±6.38	71.63±4.91	59.48±14.88	126.72±26.97	−10.47±0.32

of these compounds in the formulation were under their LD50 values (60 mL/kg and 2–4 g/kg, respectively) (23). Olive oil was used due to its ability to solubilize diarylheptanoids. The obtained nanoemulsion droplets were monodisphere and spherical with diameters ranging from 200 to 400 nm and zeta potentials less than −10 mV (24). High entrapment efficiencies and drug loading ratios of both DA1 and DA2 in the nanoemulsion were observed. By the *in situ* absorption, there were no changes in the concentrations of phenol red in the after intestinal perfusate, indicating that the absorption of DA1 and DA2 from C.c-Nano and DA-Nano was not induced by a physical leakage, such as the tight-junction damage. However, the possible mechanism of this increase of intestinal absorption needed further investigation. These results suggest that nanoemulsions of these compounds can improve their absorption and bioavailability for further enhancement of their therapeutic efficiency.

The *C. comosa* extract and DAs were stable in their oil solution. However, their conventional emulsions with a mean particle size of 14–16 μm were not stable with phase separation evident within 1 h, so that an absorption test could not be performed. The stability of the nanoemulsion was much greater while the particle size of both C.c-nano and DA-nano formulations did not change as observed at 14 days. Moreover, after keeping the formulations up to 3 months, there were no changes in their physical appearance. It is noticed that the particle size of DA-Nano was twofold larger than that of the C.c-Nano emulsion (Table III). This might be because the crude extract contained more components than just two purified compounds. These components might function as co-surfactants, which were initially partitioned into the interface, reducing the energy of the interface and removing the hydrophobic parts of the surfactant from contacts with water, finally resulting in the smaller particle size of crude extract formulation than that containing of DA1 and DA2 (25). More interestingly, the smaller particle size resulted in the increased absorption of DA2 but not of DA1 (Fig. 5). The reason was unknown, but it provided the information of how particle sizes affect the absorption of DA1 and DA2.

Although higher absorption rates of diarylheptanoids were observed when formulated in nanoemulsions, a demonstration of greater bioavailability needed to be obtained. After the active compounds were absorbed from the gastrointestinal tract, their concentrations in blood were also dependent on the first pass liver metabolism as well as by post-hepatic enzymes. Further study on the metabolite pattern of the diarylheptanoids is essential. Nanoemulsions may also induce changes in the absorption pattern of the other compounds included in the crude extract. Thus, pharmacological and toxicological studies of *C. comosa* extract and diarylheptanoids formulated in nanoemulsions are also required.

Table V. *In Situ* Intestinal Absorption Rates of DA1 and DA2 in Four Formulations

	Absorption rate (μg/min/cm^2)	
Formulations	DA1	DA2
C.c-oil	0.031±0.003	0.109±0.022
DA-oil	0.028±0.006	0.034±0.002
C.c-Nano	0.329±0.015	0.519±0.026
DA-Nano	0.380±0.006	0.428±0.036

CONCLUSION

Nanoemulsions containing diarylheptanoids or *C. comosa* extract were successfully prepared by a two-step process. These nanoemulsions were uniform and had high entrapment efficiencies and drug loading ratios. The intestinal absorption

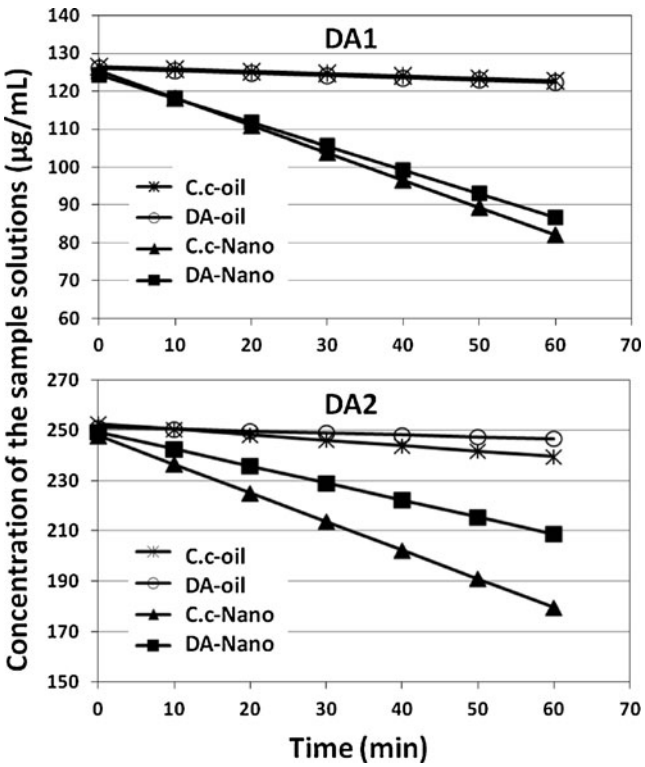


Fig. 5. Elimination rates of DA1 and DA2 of four formulations in the sample pool

rates of diarylheptanoids in nanoemulsions were five to ten times faster than when prepared as oil-based formulations which holds promise for improved therapeutic efficacy and ultimate clinical utilization.

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Conflict of Interest The authors report no declaration of interest.

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