Research Article

Topical Niosome Gel of *Zingiber cassumunar* Roxb. Extract for Anti-inflammatory Activity Enhanced Skin Permeation and Stability of Compound D

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Abstract. An extract of *Zingiber cassumunar* Roxb. (ZC) was encapsulated in niosomes of which a topical gel was formed. (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol or compound D detected by a gradient HPLC was employed as the marker and its degradation determined to follow zero-order kinetics. Niosomes significantly retarded thermal-accelerated decomposition of compound D in the gel ($p<0.05$) but did not change the activation energy of compound D. Niosomes enhanced *in vitro* permeation rate of compound D from the gel. Topical applications of ZC noisome gel gave a faster change in tail flick latency than piroxicam gel and hydrocortisone cream ($p<0.05$) while there were insignificant differences in anti-inflammatory activity up to 6 h using croton oil-induced ear edema model in mice ($p>0.05$). Thus, encapsulation of ZC extract in niosomes enhanced chemical stability and skin permeation with comparable topical anti-inflammatory effects to steroid and NSAID.

KEY WORDS: Activation energy; Compound D; Niosomal gel; Topical anti-inflammatory; *Zingiber cassumunar.*

INTRODUCTION

Niosomes, small bilayer-structured vesicles usually composed of non-ionic synthetic surfactants, are capable of functioning as depots which improve skin delivery of the encapsulated substances (1–5) including phytochemicals (6–8). Niosomal gel is usually composed of three-dimensional polymeric networks of hydrogels (9) capable of imbibing water which embeds the niosomes, thus making feasible skin applications for desirable local effects. Topical delivery and local accumulation of anti-inflammatory drugs have been shown to be improved by niosomal gels (10–13).

Rhizomes of *Zingiber cassumunar* Roxb. (ZC), family Zingiberaceae, are usually applied topically onto the skin in Thai traditional medicine and spa treatment to alleviate musculoskeletal pain, rheumatism, and localized inflammation (14–18). (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol, compound D, one of the pharmacologically active phenylbutanoids from ZC, exerts anti-inflammatory action (19,20) *via* cyclooxygenase inhibition and antioxidation (21,22) with cytochondroprotective activities which are advantageous for the treatment of arthritis (23–26). Topical products of ZC are generally prepared in emulsions or gels (27–29) from crude extracts which provide greater anti-inflammatory potency than its isolated components (21). Compound D from an alcoholic extract of ZC in a hydrogel skin patch was shown to be rapidly released, permeated through the skin with local accumulation (20). Curcumin, one of the well-studied phytochemicals, is shown to stabilize in the bilayer of niosomes by intermolecularly associating with the hydroxyl groups of cholesterol (30). The chemical structure of compound D (MW 208 g/mol) is smaller but similar to curcumin (368 g/mol); thus, it is potentially encapsulable in niosomes.

If niosomes could encapsulate phytochemicals extractable from ZC and be formulated into a niosomal gel, it is proposed that permeation and stability of compound D, employed as the representative marker, should be improved. Thus, if ZC niosomal gel improved *in vitro* skin permeation and thermal-accelerated stability of compound D, it would be compared with commercially available preparations in *in vivo* anti-inflammatory tests.

MATERIALS AND METHODS

Sorbitan monostearate (Span 60), croton oil, propylene glycol, polyethylene glycol 400 (PEG) and polyvinyl alcohol

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(PVA) from Sigma-Aldrich (USA); acetonitrile (HPLC grade) and methanol (HPLC grade) from Merck (Germany); acetic acid, triethanolamine (TEA), sodium borate, acetone and acetic acid from BDH Laboratory Supplies (England); and carbomer (Carbopol 940) from Librizol (the People's Republic of China) were used as received.

Phosphate buffer saline (PBS, Lonza, USA), disposable syringes with hypodermic thin-wall needles (0.8×25, 0.7×40 and 0.55×25 mm, Nipro®, Thailand), aluminium collapsible tubes (1.25 mm in diameter and 10 cm in length, N.R. Rama Co. Ltd., Thailand), 1% hydrocortisone cream (ATC, Thailand), 0.5% piroxicam gel (General Drugs House Co. Ltd., Thailand) and pentobarbital sodium (Nembutal® 5.47 g/ 100 ml, CEVA Sante Animal, France) were used as received.

Z. cassumunar: Preparation, Extraction and Separation

Cultivation could have effects on the quality of ZC (21,22); thus, fresh rhizomes of *Z. cassumunar* from local farms were collected from 12 to 18 months old (about 300 g/ piece) with dark brown peel and yellowish bulk. The rhizomes were peeled, sliced, dried (50±1°C, 3 days) and ground into ZC powder.

HPLC Analysis of Compound D

A gradient RP-HPLC system for determination of compound D was adopted (18,20,27,31). In brief, a gradient solvent system composed of 0.2% acetic acid in water (A) and acetonitrile (B) was varied from 70, 65, 55, 50 and 100% of A for 0–5, 5–10, 10–20, 20–30 and ≥30 min, respectively, through a RP-C$_{18}$ column (5 μm, 250×4 mm, Phenomenex, Japan) at a flow rate of 0.8 ml/min and detection at 254 nm. The HPLC validation of compound D determined limits of detection and quantitation of compound D of about 0.04 and 0.42 μg/ml, respectively, good accuracy (96.20–104.25%), precision (a coefficient of variation of less than 3.5%) and linearity with good correlation coefficient (r) ≥0.998 in the required concentration range of 1–10 μg/ml. Compound D was separated from *n*-hexane extract of ZC powder by column chromatography and eluted with a hexane-ethyl acetate gradient system followed by several more chromatograph separations on fresh silica gel columns by following a previously described method (31).

Preparation of ZC Niosomal Gel and ZC Gel

ZC powder was extracted by methanol (1:10) and filtered for ZC liquid extract which was then dried and re-dissolved with a solvent mix (ethanol/propylene glycol/water at a volume ratio of 5:14:6). PVA gel was prepared by dissolving in deionized water at a concentration of 3% *w/v*. 1.6% *w/v* carbomer gel was prepared in deionized water by adding 0.5% of TEA. The PVA gel was thoroughly mixed with the carbomer gel at a respective weight ratio of 1 to 5 to form the blank gel (the viscosity of 8–10×10^3 poise at 25°C). ZC niosomes were prepared by mixing 0.5% of span 60, 0.5% of cholesterol, 0.5% of sodium deoxycholate, 56% of propylene glycol with 42% of the ZC liquid extract and 0.5% of water with intermittent sonication for 15 min.

ZC gel, blank niosomal gel or ZC niosomal gel were formed by thoroughly mixing the ZC extract, blank niosomes or ZC niosomes, respectively, with the blank gel (2:5). The gel was filled in laminated collapsible aluminium tubes in which air volume was kept minimal. Visual observations by scanning electron microscope (SEM, Leo 1450, Germany) of ZC niosomal gel were conducted after diluting, mounting, drying and gold stubbing samples on a glass slide.

Stability Testing

Freshly prepared ZC gel or ZC niosomal gel (about 100 tubes/batch, three batches) was randomly assigned to four storage temperatures, 4, 25, 45 and 50°C at 70–75% RH. At predetermined times, two tubes of each set were sampled from the top, middle and end of each. The samples were diluted with methanol prior to being analysed by HPLC. The average compound D remaining in both gels *versus* storage durations were analysed for best fitted correlation from which the degradation kinetics and rates were derived. Their activation energies (E_a) were calculated from the slopes of the regression analyses using the Arrhenius equation (32), as follows:

$$\ln k = \ln A - \frac{E_a}{RT} \tag{1}$$

where k=degradation rate constant, A=exponential factor, E_a=activation energy, R=gas constant and T=absolute temperature (Kelvin). Stored gel and niosomal gel bases were monitored and found no peak or change.

In vitro Permeation Test

Immediately after sacrifice, full-thickness skin was excised from the dorsal part of five newborn pigs after soaking in normal saline solution at 60°C for 45 s (33,34). The skin (thickness ~0.2 mm) was mounted between donor and receptor chambers of an amber-glass 10-ml Franz diffusion cell (Crown Glass Company, USA) facing the exterior side to the donor and 0.1% albumin solution (pH 7.4) as the receptor fluid (35±1°C, 600 rpm). ZC gel or niosomal gel was applied onto the skin. Blank gel and blank niosomal gel were conducted and found no peak. The receptor fluid was sampled for HPLC analysis, and fresh receptor medium was replaced. Permeation rates of compound D were obtained by analyzing the data with Fick's law of diffusion.

Anti-inflammation and Analgesic Test in Animal Models

The Institutional Animal Ethics Committee approved the protocol (no. AEKKU 83/2556) and monitored the research conduct in full compliance with international expectations for animal care and use (35). Male ICR mice (20–40 g) from the National Laboratory Animal Center, Mahidol University, Thailand, were handled by trained personnel and caged in a controlled heating ventilating and air conditioning system at the Northeast Laboratory Animal Center as follows: 12/12-h light/dark cycle, 22–24°C, 50–70% RH, housed six mice per cage with full labels and free access to food and water. Mice were randomly assigned into groups (n=6 each), as follows: a negative control group (blank), a treatment group (ZC niosomal gel) and two positive control groups (0.5% piroxicam gel and 1% hydrocortisone cream).

Table I. Compound D Contents in Samples of Rhizomes of *Z. cassumunar* (ZC) and Products

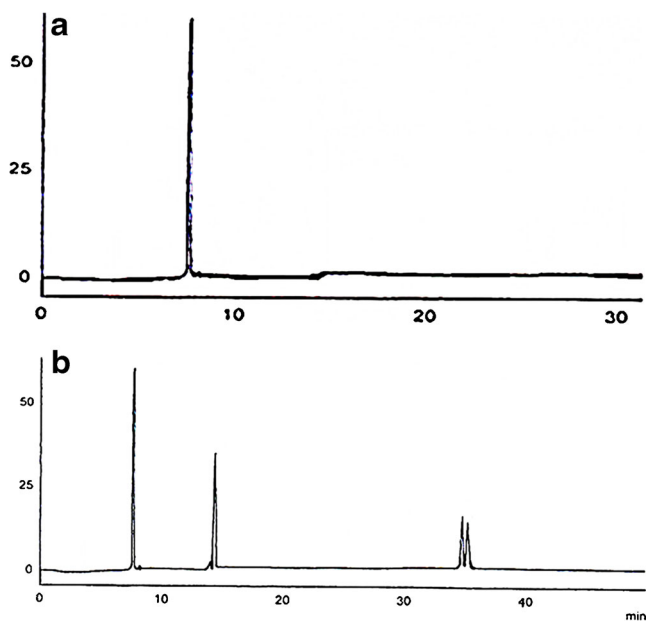| *Z. cassumunar* rhizomes (ZC)	Compound D (mg/g)
Dried powder (*n*=12 from 2 lots*)	149.5±9.1
ZC gel (*n*=6, 106% recovery)	2.2±0.1
ZC noisome gel (*n*=6, 105% recovery)	1.9±0.5

NB * lot 1 was harvested in dry season (the end of February) and lot 2 in rainy season (the end of May)

Ear edema was induced by applying 0.075 mg of croton oil (diluted in acetone) to about 1 cm^2 on the posterior part of the right ear, while the left ear received acetone alone. After evaporation of the acetone, 0.1 g of the assigned treatment was topically applied onto the same area. Ear thicknesses were determined by a digital vernier caliper (Digimatic, Mitutoyo, Japan) and used to estimate ear edema inhibition between treated and non-treated areas (36).
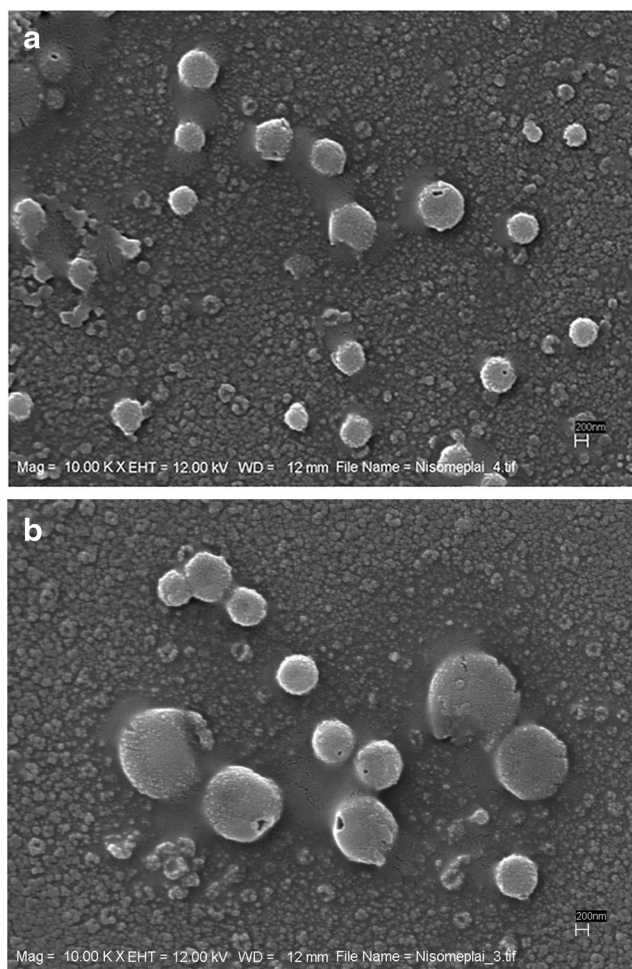
Tail flick test started 30 min after applying 0.1 g of the assigned preparation onto about 5 cm in length of the distal part of the tail of each mouse and wiping off excess. Tail flick latency was measured as the time tails contacted water at $50\pm 1°C$. Screening was performed to include only mice with baseline latency within a range of 4–7 s. Changes in tail flick latency were determined in comparison to data obtained from the relevant negative control group (37).

Statistical Analysis

Results were expressed as mean±standard deviation. Student's *t* test was used to compare significance of data between the two groups, while analysis of variance (ANOVA) and post hoc least significant difference (LSD) tests were used for
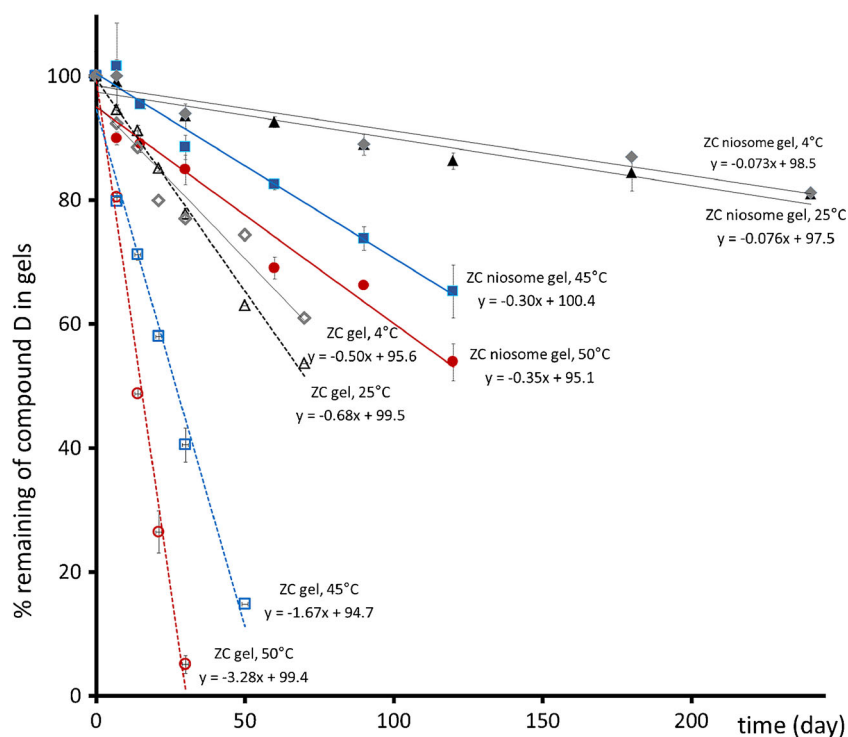

Fig. 2. Scanning electron microscopic photograph of ZC niosomal gel (**a**) freshly prepared (**b**) and stored at 50°C 70–75% RH for 60 days (10,000 dilution in water) magnification 10,000×

comparisons of >2 groups. Significances were considered at *p* values of <0.05.

RESULTS

The yields of compound D, collected within the same northeast area in Thailand, from dry and rainy seasons were 153.2 and 145.7 mg/g, respectively, which were insignificantly different (*p*>0.05), as shown in Table I. A representative chromatogram of compound D, shown in Fig. 1a and that of the ZC liquid extract, shown in Fig. 1b, indicate that compound D can be determined by the HPLC. The colour of ZC liquid extract and ZC gel was deep yellow with a strong camphoraceous smell. ZC niosomal gel was pale yellow with less smell. Both gels were viscous and easily spreadable on the skin. SEM of ZC niosomal gel showed characteristics of spherical particles of niosomes evenly distributed in water, as shown in Fig. 2a. Particle size estimation from visual scale measurement of ten frames of the SEM photographs taken from randomized samples (*n*=6 each) indicates an average particle size of 1302 nm ($P_{0.1}$=690 nm and $P_{0.9}$=1930 nm) which could be due to the interference of the gel which surrounded the

Fig. 1. HPLC chromatograms of **a** compound D with its chemical structure, and **b** methanolic extract of *Z. cassumunar* (detection at 254 nm, 250×4 mm column, gradient between 0.2% acetic acid in water (**a**) and acetonitrile (**b**) from 70, 65, 55, 50 and 100% of A for 0–5, 5–10, 10–20, 20–30 and >30 min, respectively, at a flow rate of 0.8 ml/min and detection at 254 nm

Fig. 3. Comparison of percentage of compound D remaining in ZC gel (*dashed lines*) and
ZC niosomal gel (*solid lines*) upon storage in collapsible tubes at 4°C (*grey*), 25°C (*black*),
45°C (*blue*) and 50°C (*red*)

particles. Figure 2b shows some swollen and leaked ZC niosomes after storage at 50°C for 120 days.

Figure 3 shows decrease trends of linear relationships between compound D remaining in the gels in closed containers and storage time, indicating that its degradation follows zero-order kinetics. Degradation rate constants of compound D stored at 4, 25, 45 and 50°C were 0.50, 0.68, 1.67 and 3.28% day^{-1} in ZC gel, and 0.07, 0.08, 0.30 and 0.35% day^{-1} in ZC niosomal gel, respectively.

Arrhenius plots (Fig. 4) show linear relationships between natural logarithms of degradation rates of compound D and the reciprocal of temperatures in the ZC gel ($r=-0.905$)
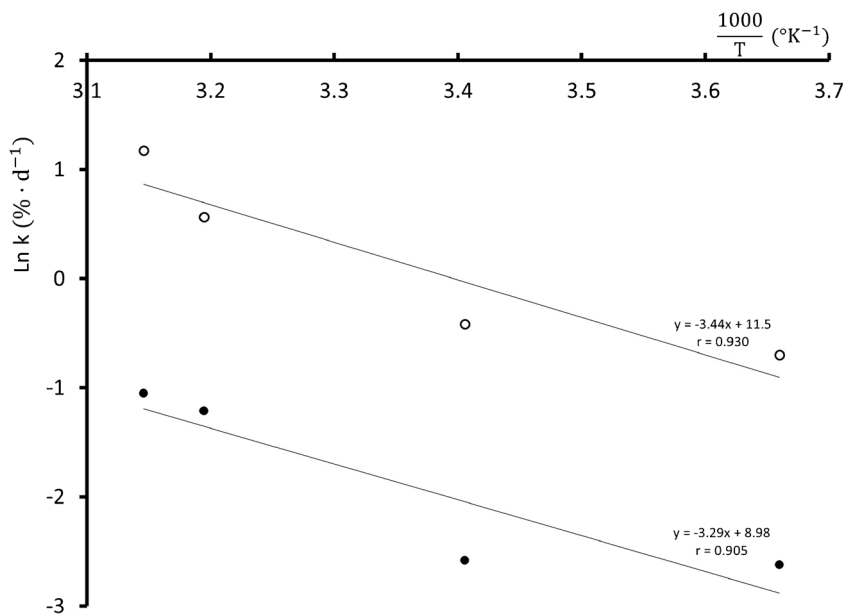
Fig. 4. Arrhenius plots between natural logarithm of degradation rate constants (day^{-1})
and the reciprocal of absolute temperatures of the storage conditions (K); ZC niosomal gel
(*empty circles*), ZC gel (*filled circles*) and the Arrhenius equation (shown in Eq. 1)
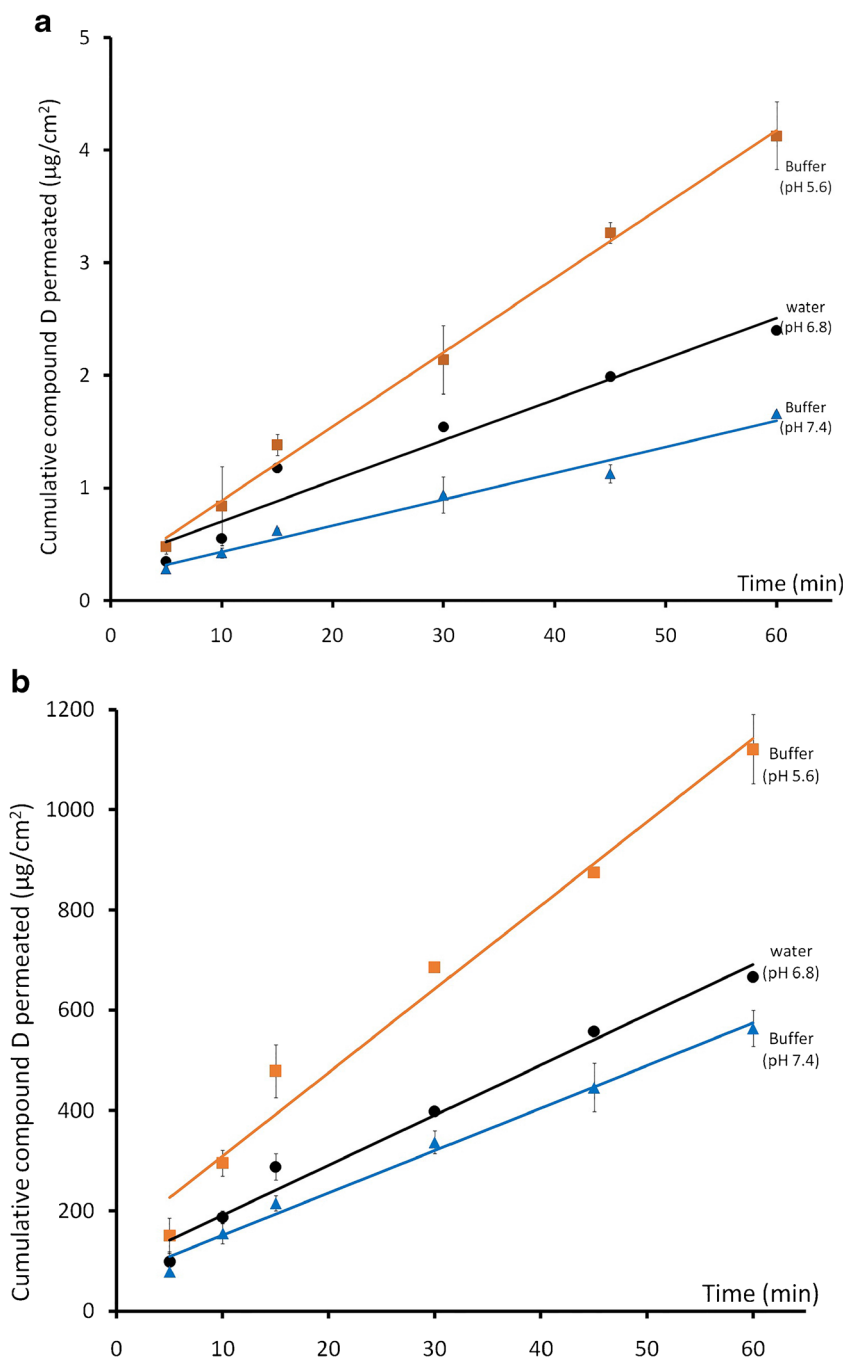
Fig. 5. _In vitro_ permeation profiles of compound D from **a** ZC gel and **b** ZC niosomal gel through porcine skin using sink conditions at 35°C into receptor solutions, citrate buffer at pH 5.6 (_orange cubes_), deionized water at pH 6.8 (_dark circles_) and phosphate buffer at pH 7.4 (_blue triangles_)

and the ZC niosomal gel ($r = -0.930$) with a parallel gap which defines about seven times higher degradation rates of compound D in ZC gel than that in ZC niosomal gel.

Compound D, from ZC gel (Fig. 5a) and ZC niosomal gel (Fig. 5b), readily and steadily permeated through porcine skin into buffers at pH 5.6 and 7.4 and water (pH 6.8). There was little or no lag time, indicating that the permeation was dominantly a result of diffusion. ZC gel provides a lower permeation of compound D than ZC niosomal gel, although both gels were prepared with the same initial concentration of compound D. Its permeation fluxes from ZC gel into water at pH 6.8 and buffers at pH 5.6 and 7.4 were 0.04, 0.07 and 0.02 µg/cm²/min, respectively ($p = 0.025$), while those from ZC niosomal gel into water of pH 5.6 and 7.4 were 10.0, 16.7 and 8.5 µg/cm²/min, respectively ($p = 0.007$). Figure 6 shows that an increase in the pH of receptor reduced compound D permeation in a linear trend, i.e. from ZC gel ($r = -0.998$) and ZC niosomal gel ($r = -0.987$).

Croton oil-induced ear edema was used to compare anti-inflammation activity of ZC niosomal gel, piroxicam
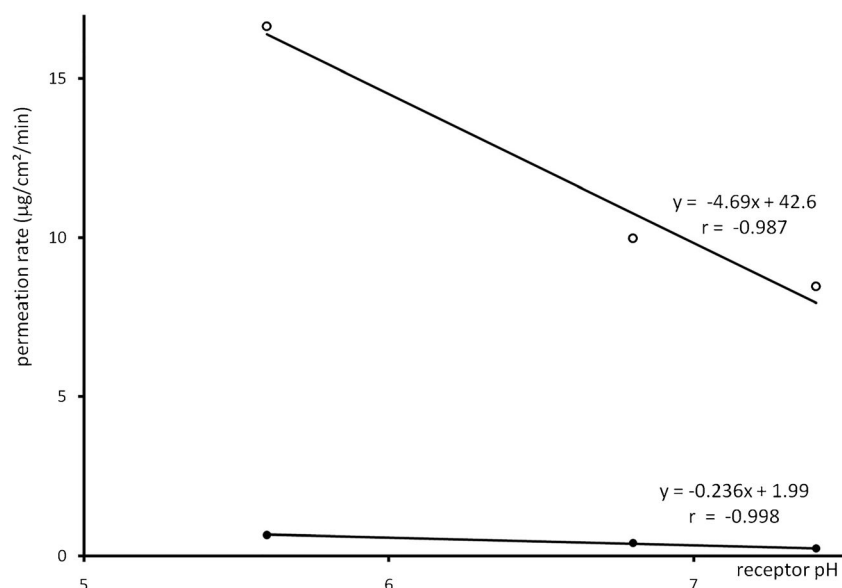
Fig. 6. Linear relationship between pH of receptor media and *in vitro* permeation flux (μg/cm²/min); ZC niosomal gel (*empty circles*) and ZC gel (*filled circles*)

gel and hydrocortisone cream and shown as ear edema and inhibition in Fig. 7a. There is no significant difference in the overall ear edema inhibition among the mice topically applied with ZC niosomal gel, piroxicam gel and hydrocortisone cream ($p<0.05$). Ear edema was gradually inhibited by ZC niosomal gel to reach its maximum inhibition of about 75% at 2 h after application and slightly decreased. Thus, topical anti-inflammatory effect of ZC noisome gel has been shown to be in line with piroxicam gel and hydrocortisone cream.

Changes in tail flick latency, which compares tolerance duration of typical tail-withdrawal reflex of the mice, before and after receiving topical applications of ZC niosomal gel, reached a maximum of about 80% at 2 h. At 1 h, ZC niosomal gel gave significantly higher (about 20%) tail flick latency changes than the other treatments ($p<0.05$). However, the change with ZC niosomal gel applications was gradually decreased after 2 h, but was not significantly different from that with piroxicam gel applications ($p>0.05$). Hydrocortisone performed the worst in this test, due to its mild analgesic activity.

DISCUSSION

Since ZC could be affected by cultivation conditions (21) or genetic diversity (22), yields of compound D are monitored to assure product quality. The samples of ZC gave about 15% yields of compound D which were insignificantly different between the collections from dry and rainy seasons ($p>0.05$). ZC gel and ZC niosomal gel were prepared to contain about 2 mg/g (or about 0.5 mg/cm²) of compound D. Despite the similar recovery, the coefficient of variation (CV) of compound D contents in ZC niosomal gel was slightly higher than ZC gel as niosomes are small vesicular dispersion in viscous gel. The particle sizes of the ZC niosomes were polydispersed with an average diameter of approximately 1 μm. Attempts to measure sizes and size distribution of the ZC niosomes by

dynamic light scattering did not obtain explainable results due to adsorption of polymers on the surface of the vesicles. Such adsorption, however, should provide 'protection' for vesicular aggregation and hold the niosomes in the gel.

ZC Gel and ZC Niosomal Gel: Stability and Permeation

It was surprising that degradation of compound D in both gels followed zero-order kinetics, indicating a concentration-independent manner. Increasing the storage temperatures increases the degradation rates of compound D in ZC gel and ZC niosomal gel. The greater the temperature dependency of degradation rate, the greater is the activation energy (E_a), estimated from the slopes of the plots in Fig. 4 by Arrhenius equation (Eq 1). E_a of compound D were, thus, estimated to be 28.6 and 27.3 kJ/mol for ZC gel and its niosomal gel, respectively. Arrhenius plots of curcumin in niosomes were also similarly parallel and comparable to solubilized form with an E_a of about 10 kJ/mol (32). Also, intermolecular interactions between curcumin and cholesterol in the niosomal bilayer reduce thermal interference to the encapsulated curcumin. Similarities in chemical structures of curcumin and compound D lead to believe that compound D would have been interacted with the components within the niosomal bilayer. Enhanced stability of compound D by niosomal encapsulation is in line with the previous report (41).

From the same gel base, compound D permeation was profoundly affected by niosomal encapsulation and, to a lesser extent, by the pH of the receptor. The amphiphile structure of compound D, hydroxyl group at one end and methoxy groups at the other end along the phenyl group, could have facilitated its diffusion through the skin, and as a result, there was no observed lag time from both formulations. This finding resembles the rapid diffusion of compound D with no lag time (20). In contrast to the first-order permeation kinetics of ZC extract in chitosan/PVA/glycerin patches with burst effects of compound D (20),
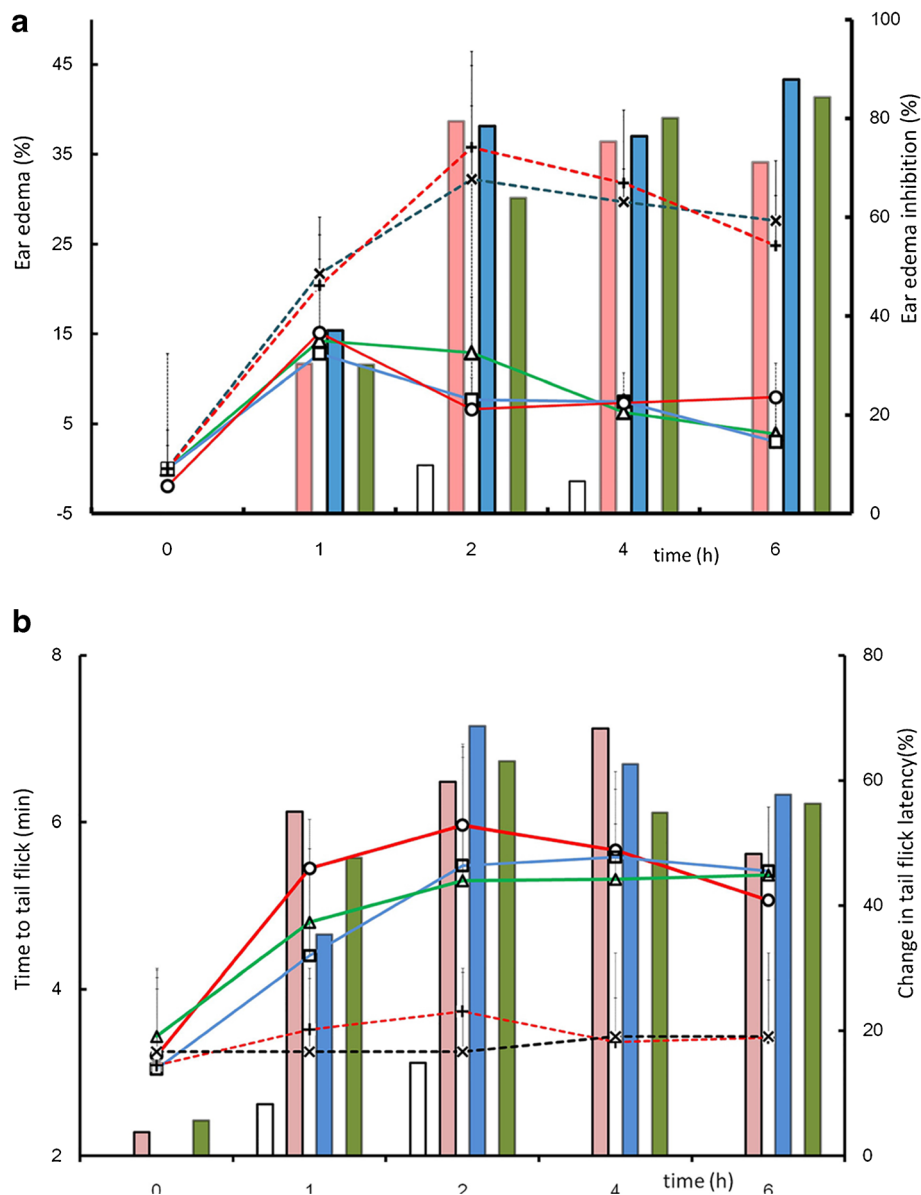
Fig. 7. Comparison of topical applications of ZC niosomal gel (*empty circles*), hydrocortisone cream (empty triangles), piroxicam gel (*empty squares*), blank niosomal gel (*plus sign*) and blank gel (*multiplication sign*) in mice with **a** croton oil-induced ear edema measured as inhibition of ear edema and **b** *bars* show time to tail flick (*n*=6 each); *error bars* represent standard deviations

our niosomal gel with encapsulated compound D provided zero-order kinetics. This leads us to suggest that diffusion of compound D was affected by the positive charges of chitosan in the patches but not our gel formula. The receptor pH and permeation fluxes of compound D from both gels were linearly correlated, as shown in Fig. 6. A receptor pH of 5.6 gave 2–3-fold higher permeation rates than at pH 7.4 which is the physiological pH. Acid condition predominates the inflamed tissue loci and triggers related immune responses (38,39). Receptor fluid at a pH of 5.6 aims to represent the skin pH of inflamed tissue pH with higher levels of hydrogen ions and may induce the hydroxyl group of the compound D to associate with water and thus facilitate diffusion and skin permeation. Enhanced permeation of compound D by niosomal encapsulation is in line with the previous report (42).

In vivo Anti-inflammatory Activity

For ethical reasons (35), ZC niosomal gel was selected to compare its activity with commercially available products and blanks in the animal studies based on *in vitro* permeation results and preliminary screening which indicated that ZC gel was insignificantly different from its blank gel. Blank niosomal gel is insignificantly different from blank gel in all tests, as shown in Fig. 7 (*p*>0.05 both), indicating no anti-inflammatory effect of the niosomes. Phenylbutanoids from ZC extract possessing anti-inflammatory and analgesic activities (17) are shown to be topically delivered by the ZC niosomal gel formulation to exert both activities in this study. 12-o-tetracanoylphorbol esters, the irritant of croton oil, cause vasodilatation within 1–2 h (36); as a consequence, increased

blood flow and vascular permeability induce cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (40). Anti-inflammatory and analgesic activities of ZC niosomal gel were observed as an immediate action with times to peak of 1 and 2 h, respectively. Secondly, both activities slightly declined following the peak times but were maintained at similar extents to the positive controls for up to 6 h ($p > 0.05$). This could be the result of an immediate permeation and acidic pH preference with the niosomal encapsulation which localized the active phenylbutanoids. This is in line with the dermal pharmacokinetic study of ZC active phytochemicals which suggests the duration of action of <2 h and a peak time at 1 h (37).

In vivo study confirms the immediate action of topically applied ZC niosomal gel at the target sites for inflammation and pain. Previous reports on *in vivo* anti-inflammatory administered crude ZC extracts by oral (17) or injection (23). The topical route provides substantial advantages over systemic administrations, for local effects. Some hydrogels can behave as burdens of skin permeation to hydrophobic compound(s). Thus, our *in vitro* and *in vivo* studies suggest that active ZC phytochemicals depend on delivery systems to exert topical activities. Herbal characteristics and yellow staining restrict the use of topical ZC products which indicates that high performance of product quality of ZC is required (20). Enhanced permeation and stability of compound D from ZC extract in a novel gel product with an emphasis on pH concerns from the present study was, thus, conclusive.

CONCLUSIONS

ZC niosomes coated with polymers with approximate sizes of 1 μm were used to form niosomal gel which contained an average compound D content of about 2 mg/g. Degradation rate of total compound D, which was affected by storage temperatures, was significantly lower with niosomal encapsulation than non-encapsulation in the same gel base ($p < 0.05$ all). *In vitro* skin permeation rate of compound D from the ZC niosomal gel was about 20-fold higher than the dissolved ZC gel. *In vivo* anti-inflammatory and analgesic testing in mice demonstrate the activities of ZC niosomal gel in comparison to piroxicam gel and hydrocortisone cream up to 6 h. Topical gel formula of herbs like *Z. cassumunar* may require innovative approaches to guarantee product quality.

ACKNOWLEDGMENTS

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