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ORIGINAL ARTICLE

Investigation of inclusion complexes of citronella oil, citronellal and citronellol with β-cyclodextrin for mosquito repellent

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Abstract The aim of this study was to prepare the inclusion complexes of citronella oil, citronellal or citronellol with β-cyclodextrin and evaluate their physicochemical properties using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). A kneading method was employed to prepare the inclusion complexes and weight ratios of each of the active substance to β-cyclodextrin were 1:1 (1:1 CPX) and 1:2 (1:2 CPX). For comparison purposes, physical mixtures of these active compounds and β-cyclodextrin were also prepared and investigated. Unlike the physical mixtures, the SEM technique revealed drastic changes in the shapes and morphologies of the particles for the inclusion complexes. Furthermore, the FTIR and DSC results seemed to reveal some interactions between the active substance and β-cyclodextrin. The o/w lotions, which contained 10% w/w citronella oil (normal citronella oil; 1:1 CPX or 1:2 CPX), were formulated using Cremophors as emulsifiers. With modified Franz diffusion cell and synthetic membrane, the release rates of citronella oil from the lotions containing the inclusion complexes were significantly lower than that from the prepared lotion containing normal citronella oil. The mosquito (*Aedes aegypti*) repellent efficacy of the lotions containing citronella oil, citronellal or citronellol (both normal and inclusion complexes) was further evaluated by human-bait technique. The highest mosquito repellent activity was observed in the formulation which contained citronella oil–β-cyclodextrin inclusion complex at weight ratio of 1:1.

Keywords Citronella oil · Citronellal · Citronellol · β-Cyclodextrin · Inclusion complex · Mosquito repellent

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Introduction

Mosquito bites cause allergic responses and transmit several life-threatening diseases such as malaria, yellow fever and dengue [1, 2]. To deter mosquitoes from biting humans or animals, the use of repellents is necessary. Based on their sources, mosquito repellents can be classified into two categories: synthetic and natural. A well known example of synthetic repellents is *N*,*N*-diethyl-*m*-toluamide or DEET which is often used in concentration ranges between 10 and 35% [1, 3]. Although DEET is effective against insects, its drawbacks, such as risks to human health and environment have led to the use of an alternative natural source [1, 4, 5]. Natural insect repellents are believed to be safer and more suitable for small children. It is generally recognized that several plant products have mosquito repellent activities

and have been traditionally used to repel mosquitoes in many regions [2, 4]. These include volatile oils extracted from a large number of aromatic plants such as *Cymbopogon nardus*, *Eucalyptus maculate* and *Mentha piperita* [2, 5]. The mechanism of action of volatile oils is to provide vapor barrier that can deter or repel the mosquitoes from coming into contact with the skin of humans and animals [5]. Citronella oil, extracted from *Cymbopogon* species like *C. nardus* L. and *Cymbopogon winterianus*, has long been used as an insect repellent since its discovery in 1910 [1]. Citronella oil, a pale to dark yellow liquid, is obtained by steam distillation of dried *Cymbopogon* plants. Commercially, there are two main sources of citronella oil: Sri Lanka (Ceylon) and Java origins [6]. It is relatively safe (GRAS status), comparable effective and approved by customers [5]. Nevertheless, neat citronella oil should not be directly applied onto the skin since it may cause skin irritation and sensitization. The dermal LD_{50} of citronella oil is reported to be between 3.4 and 6.7 g/kg in rabbit [6]. Recently, citronella oil has been registered as insect repellent ingredients for skin application by the US Environmental Protection Agency (US EPA) [1, 5]. It has been reported that the effective concentration ranges of citronella oil vary from 0.05 to 25% (w/v) alone or in combination with other natural or commercial insect repellents. Citronella oil contains more than a dozen terpene components, such as geraniol, citronellal and citronellol [6], which are associated with insect repellent properties [3]. As with many volatile oil-based repellents, the crucial problem of citronella oil is short-lasting protection, which is related to its high volatility [5]. Generally, the average protection periods of botanical repellents tend to be less than 23 min [7]. In view of this, the reduction of volatility to achieve prolonged repellency duration is required for citronella oil-based formulations.

Up until now, several strategies have been employed to increase the repellent efficiency of volatile oils. These include the use of polymer mixtures, microcapsules, nanoemulsions and some fixative substances, such as vanillin, coconut oil, mineral oil and mustard [5, 8–11]. For example, Sakulka and co-workers [11] prepared citronella oil nanoemulsions by high pressure homogenization at varying amounts of surfactant (Montanov® 82) and glycerol. It was found that droplet size and concentrations of surfactant and glycerol influenced the release characteristics of citronella oil. Additionally, the mosquito protection times were related with the release rates of citronella oil. The decreased release rates resulted in a prolonged protection period. In another study, the o/w type chitosan encapsulated citronella oil microcapsules were prepared by a modified orifice method. Citronella oil (10% (v/v)) was the core material and chitosan, a naturally occurring polysaccharide, was the wall membrane. By applying the thermal pretreatment of microcapsules, the sustained release of citronella oil was achieved. The sustaining effect of the oil was found to increase when the treatment time increased. These sustained properties were attributed to the heat shrinking property of chitosan [8]. Nevertheless, the mosquito repellency was not determined in their study.

Forming a complex with cyclodextrins is another technique used to modify the release of volatile components [12]. Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and internal hydrophobic cavities which are able to form inclusion complexes with a large number of molecules [13, 14]. Several methods have been applied to prepare the inclusion complexes between cyclodextrins and guest molecules. These include co-precipitation, slurry complexation, paste complexation, damp mixing and heating, extrusion and dry mixing. Details of these methods are given elsewhere [15]. Based on the number of D-glucopyranose units, three types of naturally occurring cyclodextrins have been classified; α-cyclodextrin (six units), β-cyclodextrin (seven units) and γ-cyclodextrin (eight units) [15, 16]. These cyclodextrins have different ring sizes and physicochemical properties [14]. For example, the cavity diameter of α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin is about 5.0, 6.3 and 8.0 Angstroms, respectively [16]. Among the natural cyclodextrins, β-cyclodextrin is the most popular since it is not costly and has a wide range of applications in many areas including medicals, pharmaceutics, cosmetics and foods [15, 16]. In addition to oral application, the use of β-cyclodextrin in topical products is well recognized. Furthermore, β-cyclodextrin does not cause skin irritation [14]. For these reasons, β-cyclodextrin was selected to create the inclusion complex with citronella oil in an attempt to reduce its volatility and to prolong its insect repellent activity. Although several researchers have studied the complexation between cyclodextrins and citronella oil, only few works have investigated the insect repellent activity of the dermatological products. Besides citronella oil, the complexation of major components of citronella oil (citronellal and citronellol) was also evaluated in the current study.

The aim of this study was to prepare the inclusion complexes of citronella oil and its major components with β-cyclodextrin using the kneading method (paste complexation). Physical mixtures of the active substance and β-cyclodextrin were also prepared for comparative evaluation. The characterizations of the inclusion complex and the physical mixture products were performed using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The in vitro release property of citronella oil (both normal and inclusion complexes) from oil-in-water (o/w) lotions were investigated by modified Franz

diffusion cell technique. Finally, the mosquito repellency of lotions containing these actives was tested by the in vivo human-bait technique.

Materials and methods

Materials

Citronella oil, obtained by steam distillation of *C. winterianus*, was purchased from Thai-China Flavours and Fragrances Industry Co., Ltd (Ayutthaya, Thailand). (±)-Citronellal was obtained from Aldrich Chemical Company (USA). Citronellol was supplied by Fluka (Switzerland). β-Cyclodextrin was obtained from Wacker Chemie GmbH (Germany). Absolute ethanol was obtained from Merck KGaA (Germany). Cremophore A6, Cremophore A25, stearic acid, Eutanol-G, cetyl alcohol, Glycerine, and Uniphen P-23 (preservative) were supplied by P.C. Drug Center Co., Ltd. (Bangkok, Thailand). Cellulose acetate membrane (Spectra/Por®3 dialysis tubing, MWCO 3500) was purchased from Spectrum Laboratories Inc. (US & Canada). All chemicals were of analytical grade and were used without further purification.

Methods

Gas chromatography–mass spectrometry analysis (GC–MS)

GC–MS analysis was performed on a HP5890 Gas Chromatograph-HP5972 Mass Selective Detector (Hewlett Packard, Palo Alto, California, USA), equipped with electron ionization and a HP-Innowax (30 m × 0.25 mm i.d.; film thickness 0.25 μm) capillary column (Hewlett Packard). The inlet temperature and transferline temperature was 260 °C. The carrier gas, helium, had a flow rate of 1 mL/min and the solvent delay time was 3.0 min. Mass range was from 35 to 500 amu. The column oven temperature was programmed as follows: initial temperature 80 °C, 2 min; ramp to 150 °C at 3 °C/min; ramp to 185 °C at 2 °C/min and ramp to 250 °C at 10 °C/min. The components of citronella oil were identified using Wiley 275.L Mass Spectra Database Library. GC peak areas were used to determine the percentage compositions of citronella oil.

Preparation of inclusion complexes and physical mixtures

The inclusion complexes of citronella oil with β-cyclodextrin at weight ratios of 1:1 and 1:2 (guest:host) were prepared by the kneading method. β-Cyclodextrin was accurately weighed and transferred into a porcelain mortar. Small amounts of purified water were dropped onto the cyclodextrin, and then mixed with a pestle until a paste was obtained. Then, citronella oil was incorporated into the paste and mixed thoroughly until homogeneous. In order to reduce the loss of volatile oil, the prepared inclusion complexes were dried under low temperature (20 °C). Additionally, they were placed in a suitable container and covered with an aluminum foil with a few holes in it for drying. The physical mixtures in the same ratios as the inclusion complexes were prepared by gently mixing citronella oil with β-cyclodextrin for 2 min using a porcelain mortar and pestle. The inclusion complexes and physical mixtures of citronellal or citronellol, the major components of citronella oil, were also prepared. The obtained products were stored in well-closed containers, protected from light and kept in a cool place prior to use.

Investigating the physicochemical properties of inclusion complexes

SEM The morphology of β-cyclodextrin, inclusion complexes and physical mixtures were assessed by a Quanta 400 scanning electron microscope (FEI Company, Czech Republic) fitted with an Everhart-Thornley detector. The specimens were coated with gold sputtering (SPI Supplies, USA), and examined at 15 kV under high vacuum conditions ($<1.3 \times 10^{-2}$ Pa).

FTIR Infrared spectra of the samples (pure compounds, inclusion complexes and physical mixtures) were analyzed using a Perkin-Elmer model Spectrum One FT-IR Spectrometer (Perkin-Elmer Co. Ltd., USA). The samples were ground and mixed thoroughly with KBr. Then, KBr discs were produced by compressing the mixed samples in a hydraulic press. The transmittance spectra were obtained in the range of 4,000–400 cm^{-1} with a spectrum resolution of 4 cm^{-1}. The recorded spectra were the result of averaging 8 scans.

DSC DSC thermograms of the samples were recorded on a Perkin-Elmer DSC model 7 (Perkin-Elmer Co. Ltd., USA). Samples were accurately weighed into Perkin-Elmer DSC sample pans, hermitically sealed and heated at a rate of 20 °C/min. The heating range was between 30 (room temperature) and 200 °C under a nitrogen flow of 150 mL/min.

Evaluation of evaporation of inclusion complexes of citronella oil

The evaporation of normal citronella oil and citronella oil–β-cyclodextrin inclusion complexes were determined using Mettler LP 16 Infrared Dyer and Moisture Analyzer equipped with Mettler PM 300 automatic balance (Mettler-

Toledo AG, Switzerland). The test samples with equivalent amount of citronella oil were placed in the open-lid containers and heated at 50, 90 and 120 °C for 50 min. The weight loss of the samples was measured and recorded.

Preparation of citronella oil lotions

Citronella oil lotions were formulated in the form of o/w emulsions containing 10% w/w of citronella oil (or equivalent to 10% citronella oil in the case of the inclusion complexes). The compositions of the lotions are shown in Table 1.

The lotions were prepared by the beaker method. The oil phase ingredients were melted together to about 70 °C; meanwhile the water phase ingredients were heated together to about 75 °C. The water phase was then added to the oil phase slowly under constant stirring. Upon cooling to about 40 °C, the active ingredient was added with continuous stirring until uniform. The prepared lotions were stored in a cool place until used.

Physical property study of the prepared lotions

Appearance, pH and viscosity of these prepared lotions (F2–F4) and the commercial lotion (F1) were investigated by means of the following procedure:

Appearance The appearance of the lotions was examined with naked eyes. These included color, phase separation, creaming and uniformity.

Table 1 Ingredients for o/w lotions containing citronella oil (normal and inclusion complex) as active ingredient

Ingredients (% w/w)	Formulation code[c]		
	F2	F3	F4
Cremophore A6[a] (emulsifier)	3	3	3
Cremophore A25[a] (emulsifier)	3	3	3
Stearic acid[a] (stiffening agent)	6	6	6
Eutanol-G[a] (emollient and lubricant)	5	5	5
Cetyl alcohol[a] (stiffening agent)	2	2	2
Glycerine[b] (humectant)	5	5	5
Uniphen P-23[b] (preservative)	1	1	1
Citronella oil	10	–	–
Citronella oil:βCD (1:1) complex	–	20	–
Citronella oil:βCD (1:2) complex	–	–	30
Purified water[b] to	100	100	100

[a] Oil phase ingredients

[b] Water phase ingredients

[c] A commercial lotion containing 10% w/w citronella oil was coded as F1 (solution-type lotion)

pH measurement The pH of the lotions was determined by a digital pH meter (model Seven Easy S20, Mettler Toledo Co., Ltd., USA). The measurement was conducted in triplicate at room temperature (31.0 ± 1.0 °C).

Viscosity measurement The viscosity of the formulations was determined in triplicate at room temperature (31.0 ± 1.0 °C) using a bob-cup Brookfield viscometer (model DV-III ultra, Brookfield Engineering Laboratories Inc., Stoughton, MA, USA). Brookfield Rheocalc software (version V 3.1-1) was used to control the viscometer. In the current study, the viscosity at the highest %torque was reported.

In vitro release study of citronella oil

The in vitro release of citronella oil from the formulations F1–F4 was investigated using Modified Franz diffusion cell. The apparatus was set at 37 °C by circulating water bath, providing the membrane surface temperature of 32 °C. The vertical diffusion cell consisted of donor and receptor compartments. A diffusion surface area was 1.77 cm^2. A synthetic membrane (Spectra/Por®3) was employed as a semipermeable membrane separating the donor compartment from the receptor compartment. The membrane (dialysis tubing) was cut into 4.5 × 4.5 cm and boiled in distilled water to remove any wax coated on the membrane. The obtained membrane was fully hydrated and stored in a cool place until used (within a week). The receptor fluid, a mixture of water and absolute ethanol (1:1 v/v), was degassed and filled into the receptor compartment (10 mL). Absolute ethanol was employed to solubilize citronella oil. The receptor solution was stirred at 200 rpm with Teflon-coated magnetic bars and magnetic stirrer (Variomag Telemodul 40S, Germany). The hydrated membrane was placed onto the receptor compartment followed by the donor compartment and secured with a clamp. After equilibrating the membrane for 30 min, a weighed sample (equivalent to 0.05 g citronella oil) was placed on the membrane in the donor compartment and covered to prevent evaporation. At designated time intervals (1, 2, 3, 4, 6, 8, 10 and 24 h), 1 mL aliquot was taken from the receptor phase and replaced immediately with the degassed receptor fluid. The amount of citronella oil in the collected receptor solution was then assayed spectrophotometrically at sharp peak of 229 nm by UV visible spectrophotometer (Spectronic Genesys 5, Thermo Fisher Scientific, USA). The analysis of amount of volatile oil released was based on the experiment done by Maji et al. [10], who measured the amount of *Zanthoxylum limonella* oil released from microcapsules by means of UV technique.

The sharp peak of citronella oil was determined by scanning a known concentration of citronella oil in the mixture of water:ethanol (receptor fluid) in the range of 200–400 nm. In the case of calibration curve, citronella oil standard solutions of concentration in the range of 50–500 μg/mL were prepared with the mixture of water:ethanol. The calibration curve was fitted with straight line equation obtained by using linear regression analysis. The concentration of citronella oil in the withdrawn aliquots was calculated by means of calibration curve. Blank lotion samples were also run at the same time to check for any interferences caused by the lotion bases. The cumulative amount of citronella oil released (Q_t) from the formulations was calculated from the following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i \quad (1)$$

where C_t is the concentration of citronella oil of the receptor fluid at each sampling time, C_i is the concentration of citronella oil of the ith sample, and V_r and V_s are the volumes of the receptor fluid and the sampling solution, respectively. To construct the in vitro release profile, the cumulative amount released of citronella oil per unit area was plotted against time and the release rate was calculated from the slope of the linear portion of the graph.

Mosquito repellent test

In this study, the lotion formulations containing normal or the inclusion complexes of citronella oil, citronellal or citronellol were tested for their mosquito repellency against *Aedes aegypti* using the human bait technique (arm-in cage assay). This study was approved and conducted by the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. The test procedure was based on the standard method of Tawatsin et al. [17]. The repellency test was conducted in a 6 × 6 × 3 m room at 25–29 °C with relative humidity of 60–80%. The period of repellency test was up to 7 h. Prior to the test, hands and arms of three human volunteers ($n = 3$) were washed and cleaned with water and detergent and thoroughly dried. For testing, one forearm of each volunteer was marked with a permanent marker, creating an area of 3 × 10 cm whereas another one was unmarked and used as a control. A marked area of each forearm of each volunteer was thoroughly applied with the test formulation (0.1 g). Each arm (both treated and control arms) of each volunteer was covered with paper sleeve with a 3 × 10 cm exposed area.

After the treatment, each volunteer put the treated arm into a mosquito cage (30 × 30 × 30 cm) containing 250 host seeking female *A. aegypti* mosquitoes for the first 3 min exposure period of every 30 min interval. The untreated arm (control) was exposed to mosquitoes in the cage for 1 min. The number of biting mosquitoes on the exposed area of both arms was recorded at each interval. The repellency test was performed every 30 min and continued until at least two bites occurred on the treated area of the arm in a 3 min exposure period. The protection time was the time between application of the test formulation and the second successive bites.

Statistical analysis

One-way analysis of variance (ANOVA) and Student's t test were used to examine the statistically significant differences. A p-value of less than 0.05 was considered significantly important.

Results and discussion

Identification of citronella oil

The compositions of citronella oil (*C. winterianus*) (Java type) determined by GC–MS are summarized in Table 2. Citronella oil was found to contain a mixture of several terpenes. The most abundant component was citronellal (30.59%) followed by citronellol (19.30%) and geraniol (18.17%). It was noted that the major constituents of citronella oil used in the current study were different from those published in the literature [6]. For example, geraniol (17.0%) and limonene (11.3%) were the major components found in Ceylon-type citronella oil [6]. These differences

Table 2 Identification of citronella oil components by GC–MS technique

t_R (min)	Compound[a]	Area (%)
4.12	Limonene	4.59
11.98	Citronellal	30.59
12.41	β-Bourbonene	0.149
13.10	L-Linalool	1.20
13.86	Isopulegol 3	1.90
14.48	β-Elemene	3.65
17.00	Citronellyl propionate	4.43
21.13	Citronellol	19.30
24.10	Geraniol	18.17
31.17	Elemol	3.34
34.04	Eugenol	2.07
34.92	δ-Cadinol	0.22
35.59	α-Eudesmol	0.47
41.35	Farnesol	0.64

[a] Wiley 275.L Mass Spectra Database was used to identify the compounds

may be due to the variation in geographical location, weather conditions and soil types [18].

Both citronellal and citronellol have been found to possess mosquito repellent activities [5]. In addition to *Cymbopogon* plants, citronellal and citronellol are also the constituents of eucalyptus oil extracted from the leaves of *Eucalyptus citriodora* [19]. It has been reported that the bacteriostatic activity of eucalyptus oil towards *Staphylococcus aureus* is due to the synergistic effects of these two compounds [19]. With respect to safety, citronellal and citronellol may cause skin irritation when applied directly onto the skin. Citronellol is considered as being GRAS (Generally Recognized as Safe for food use) by the US EPA. Citronellal is the acyclic terpene aldehyde, whereas citronellol is the acyclic terpene alcohol. Both of them are volatile fragrance compounds that possess certain properties as summarized in Table 3.

Similarly to citronella oil, the controlled delivery of these fragrance terpenes is needed to improve the mosquito repellent efficiency. In the current study, the two major components of citronella oil, citronellal and citronellol, were also used to form the inclusion complexes with β-cyclodextrin. As with citronella oil–β-cyclodextrin inclusion complexes, the formation of these complexes was also analyzed using SEM, FTIR and DSC techniques.

Appearances of inclusion complexes

The inclusion complexes of citronella oil and β-cyclodextrin at both weight ratios were coarse powders with a citrus like odor. The color of the complexes was somewhat different, yellow-white at weight ratio 1:1, and white at weight ratio 1:2. The difference in their colors was due to the amount of β-cyclodextrin used. Similarly, the inclusion complex products between citronellal or citronellol and β-cyclodextrin were white coarse powders with distinctive citrus scents.

Investigation of inclusion complexes

The complexation between citronella oil, citronellal or citronellol was investigated by using SEM, FTIR and DSC techniques.

SEM analysis

The surface morphologies of the products were further investigated by means of SEM. Figure 1 shows the SEM images of pure β-cyclodextrin at different magnifications. The β-cyclodextrin was composed of different sizes of rectangular shaped crystals. In addition, there were small particles that adhered to the surfaces of the crystals.

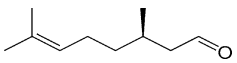
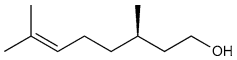
For citronella oil, citronellal and citronellol, the SEM pictures of the inclusion complex and physical mixture products are shown in Fig. 2.

As seen from Fig. 2a, there were drastic changes in particle shapes and original morphologies of the inclusion complex products. The complexation between citronella oil and β-cyclodextrin at both weight ratios appeared as agglomerates. The degree of agglomeration seemed to increase with increasing weight ratio from 1:1 to 1:2. In contrast, the particle shapes and morphologies of the corresponding physical mixtures at both weight ratios were similar to those of β-cyclodextrin. In addition, there were no observable changes when the weight ratios increased from 1:1 to 1:2. The particle sizes of the physical mixtures were much larger than those of the inclusion complex products.

The inclusion complexes and the physical mixtures of citronellal and β-cyclodextrin at weight ratios of 1:1 and 1:2 are shown in Fig. 2b. Unlike physical mixtures, the drastic changes in particle shapes and original morphologies were clearly observed in the case of citronellal–β-cyclodextrin inclusion complex products. Agglomerations were observed in the case of the inclusion complexes. Increasing the weight ratios from 1:1 to 1:2 resulted in increase of the size of the agglomerated particles. In the case of the physical mixtures at both weight ratios, the particle shapes and morphologies were found to similar to those of β-cyclodextrin. No observable changes were noted when the weight ratios increased from 1:1 to 1:2. As with citronella oil, there were significant differences of the particle sizes between the inclusion complexes and the physical mixture products.

Figure 2c shows SEM images of the inclusion complexes and the physical mixtures of citronellol and

Table 3 Characteristics of citronellal and citronellol [20]

Name	Chemical name	Structure	Formula	Molecular weight	Appearance
Citronellal	3,7-Dimethyl-6-octenal		$C_{10}H_{18}O$	154.24	Colorless aromatic liquid
Citronellol	3,7-Dimethyl-6-octen-1-ol		$C_{10}H_{20}O$	156.26	Colorless aromatic liquid

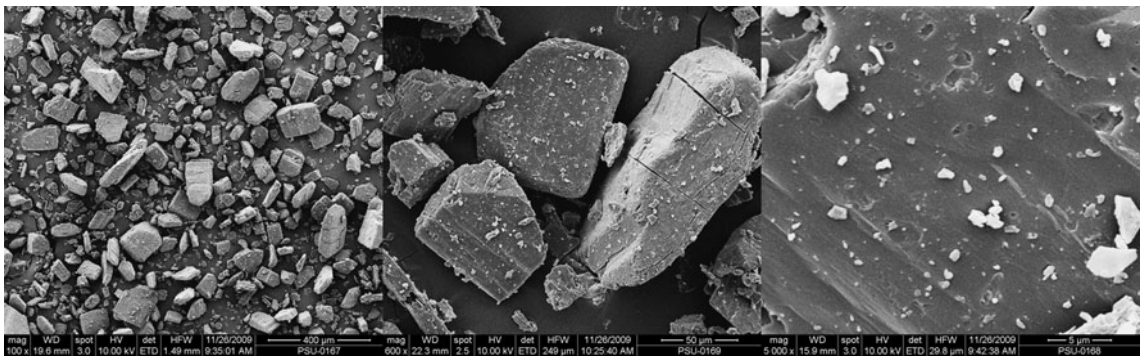


Fig. 1 SEM photographs of β-cyclodextrin, magnification $\times$100 (*left*); magnification $\times$600 (*middle*); magnification $\times$5000 (*right*)

β-cyclodextrin at weight ratios of 1:1 and 1:2. The inclusion complexes of citronellol appeared as agglomerates similar to those produced by citronellal. No changes in shapes and morphologies of the β-cyclodextrin were observed in the case of the physical mixtures when compared with pure β-cyclodextrin.

Based on the aforementioned results, the formation of inclusion complexes between citronella oil, citronellal or citronellol and β-cyclodextrin was reasonably demonstrated by the SEM technique.

FTIR analysis

To detect the inclusion complexes, FTIR was another method used in the current study. Changes in the characteristic bands of pure compounds could indicate the existence of complexes [21].

It can be seen in Fig. 3a that pure citronella oil showed intense bands at several wavenumbers. Such bands appeared at 3413, 2923, 2721, 1727, 1671, 1645, 1516, 1453, 1378, 1235, 1016 and 738 cm^{-1}, corresponding to chemical functional groups of various terpenes presented in the oil (see Table 2). Citronella oil contained mainly citronellal, citronellol and geraniol and accordingly, these components, in particular citronellal, dominated the vibration spectra of the oil. As a result, the IR spectra of citronella oil were characterized by principle absorption peaks at 3413 (O–H stretch), 2721 (aldehydic C–H stretch), 1727 (C=O stretch), 1645 (O–H bend), 1378 (deformation of C–O–H group) and 1016 cm^{-1} (C–O stretch). FTIR spectra of pure β-cyclodextrin showed the intense vibration bands at 3380, 2923, 1645, 1156, 1028 and 762 cm^{-1}. These findings are comparatively in agreement with the literature data [22]. Details of each band position are presented in Table 4.

The FTIR spectra of the inclusion complexes were compared to those of the physical mixtures and the pure compounds (citronella oil, or β-cyclodextrin). It was observed that some minor differences in the IR spectra appeared. Both inclusion complex and physical mixture products weaken some bands of pure citronella oil such as 1671 and 1016 cm^{-1}. A higher shift of O–H bending (1645 cm^{-1}) was observed for physical mixtures, 1:1 PM and 1:2, whereas a lower shift was obtained for the inclusion complexes, 1:1 CPX and 1:2 CPX. In the case of the inclusion complexes, there was a shift of wavenumber corresponding to O–H stretching for citronella oil (3413 cm^{-1}). As demonstrated in Fig. 3a, the wavenumbers for 1:1 CPX and 1:2 CPX were shifted to 3339 and 3391 cm^{-1}, respectively. Overall, the IR spectra changes may suggest the interaction between citronella oil and β-cyclodextrin.

FTIR spectra of pure citronellal, pure β-cyclodextrin together with their physical mixtures and inclusion complex products are shown in Fig. 3b. In the case of citronellal, there were six intense bands occurring at 2916, 2716, 1727, 1454, 1378 and 825 cm^{-1}. The major spectral features were the stretching mode of C=O at 1727 cm^{-1} and the aldehydic C–H stretching at 2716 cm^{-1}. The stretching vibration of C=C at about 1630–1660 cm^{-1} was not observed. It has been reported that the Raman spectra could give this stretching characteristic band [19]. By comparing the spectra of pure β-cyclodextrin with those of the obtained products (inclusion complexes and physical mixtures), there were lower shifts of the O–H stretching and O–H bending in the inclusion complexes. However, higher shifts of the same bands were recorded in the physical mixtures. With respect to the aldehydic C–H stretching of pure citronellal (2716 cm^{-1}), the wavenumbers for 1:1 CPX, 1:2 CPX, 1:1 PM and 1:2 PM were shifted to 2712, 2721, 2712, and 2721 cm^{-1}, respectively. It was noted that lower shifts were observed when the weight ratio between citronellal and β-cyclodextrin was 1:1 but higher shifts were recorded when the weight ratio increased to 1:2. In addition, the peak position of citronellal at 825 cm^{-1} disappeared in both inclusion complexes and physical mixtures, indicating that these products weaken this band. However, the shift of C=O stretching at 1727 cm^{-1} was not observed in all products. Based on these results, it may be concluded that there were

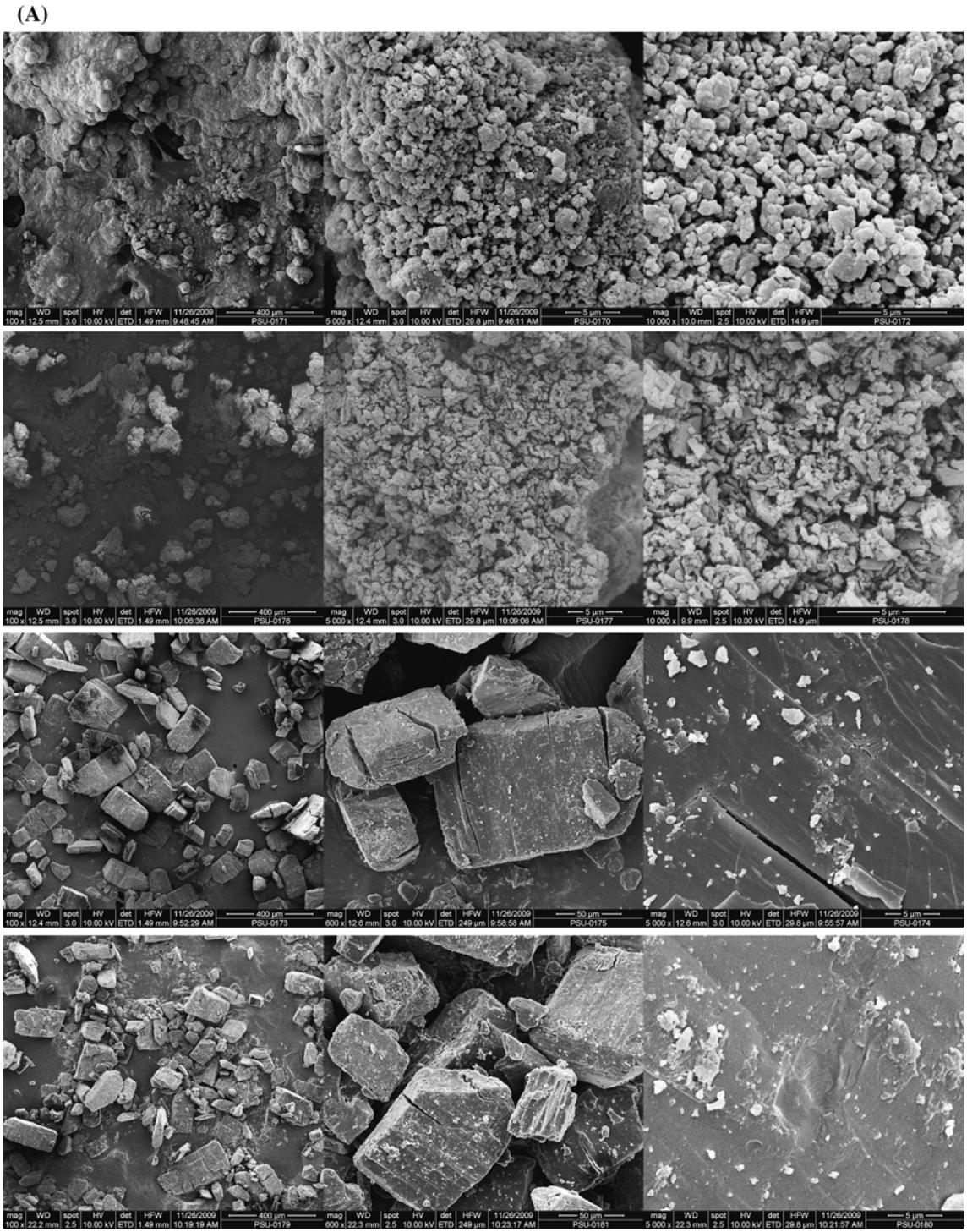


Fig. 2 SEM photographs of inclusion complexes (*rows 1 and 2*: *row 1* weight ratio 1:1, *row 2* weight ratio 1:2) and physical mixtures (*rows 3 and 4*: *row 3* weight ratio 1:1, *row 4* weight ratio 1:2); citronella oil (**a**), citronellal (**b**) and citronellol (**c**)

some differences in the FITR spectra of the inclusion complexes, the physical mixtures and the pure components. The spectral changes observed in the inclusion complexes may suggest a chemical interaction between citronellal and β-cyclodextrin.

Intensive signals of citronellol were observed at 3341, 2925, 2725, 1455, 1378, 1058, 830 and 738 cm^{-1} (see Fig. 3c). The major peaks at 3341 cm^{-1}, of O–H stretching, and 1058 cm^{-1}, of C–O stretching, and 1378 cm^{-1}, due to deformation of the C–O–H group [19] were the most

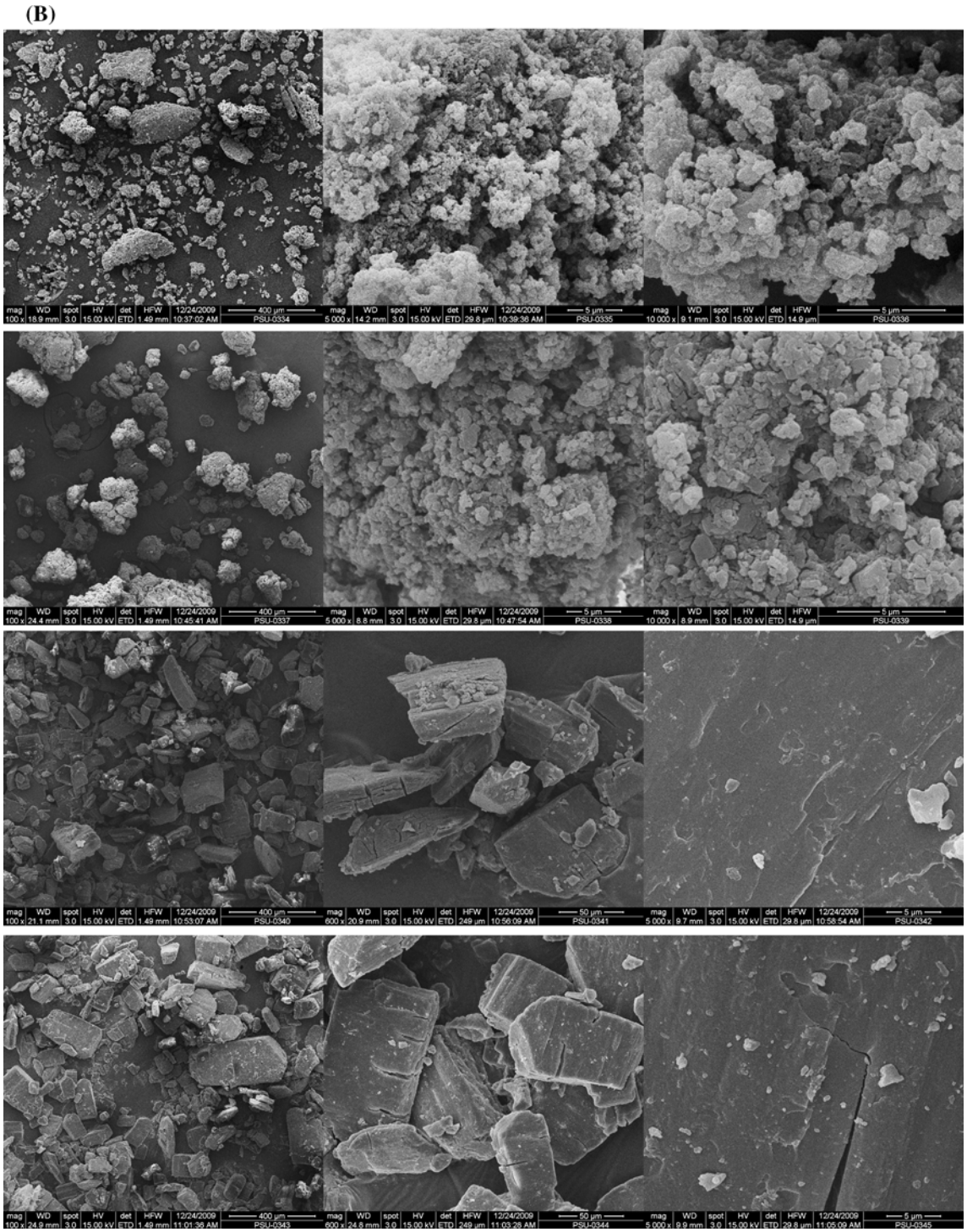


Fig. 2 continued

important characteristics of citronellol. Again, the absorption peak of C=C stretching was not recorded. As shown in Fig. 3c, the wavenumbers for 1:1 CPX, 1:2 CPX, 1:1 PM and 1:2 PM were shifted to 3359, 3354, 3354 and 3353 cm^{-1}, respectively. These results indicate a H-bonding interaction between citronellol and β-cyclodextrin. It was noted that there was an overlapping effect in this region, as demonstrated by the intense band at 3380 cm^{-1} for β-cyclodextrin. This band corresponded to O–H stretching. In addition, the position of the C–H stretching at

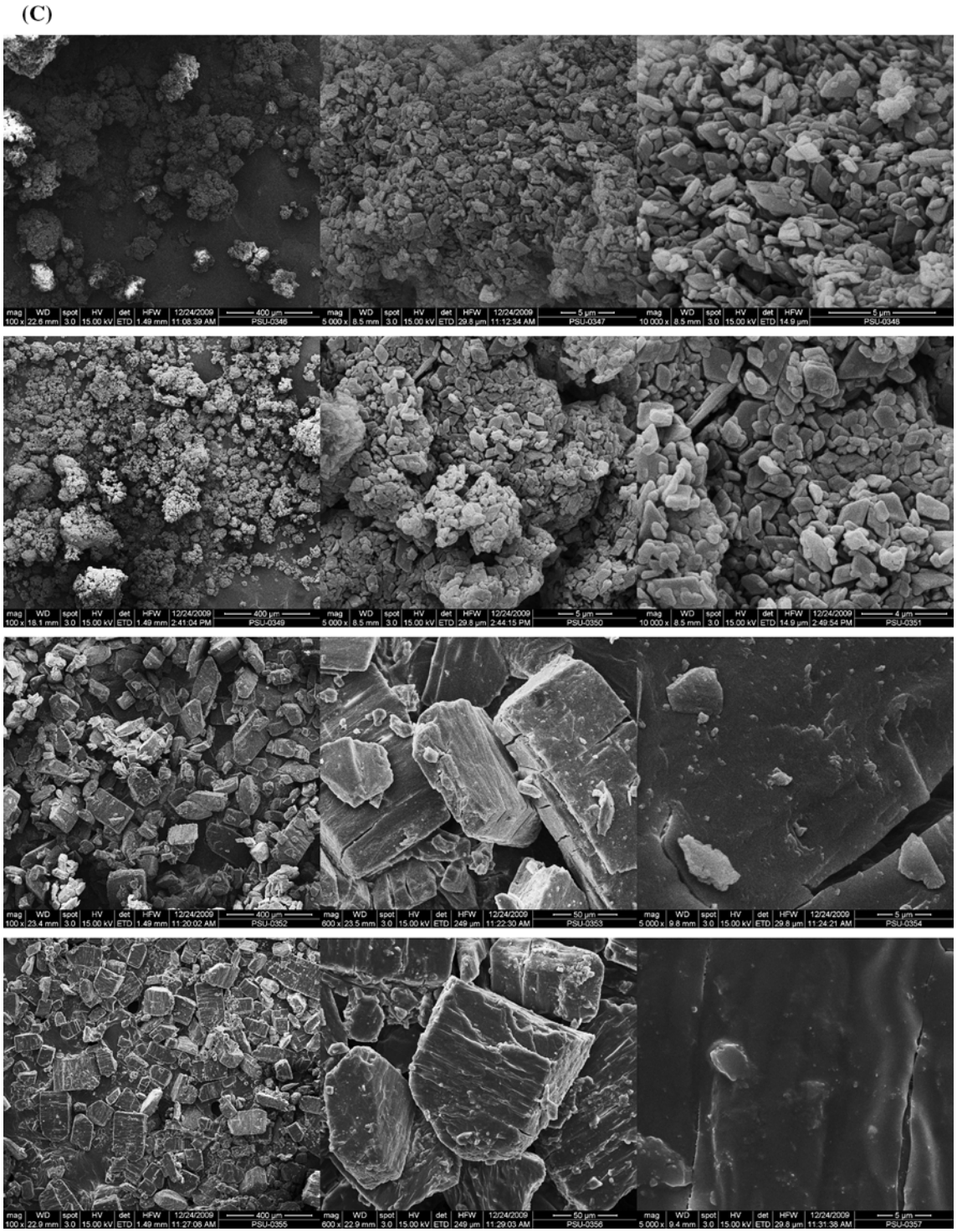


Fig. 2 continued

2925 cm^{-1} for pure citronellol was shifted to lower frequency at 2919 cm^{-1} in the case of 1:2 CPX. The shifts in these wavenumbers may indicate an interaction between citronellol and β-cyclodextrin in the inclusion complex products.

DSC analysis

Another technique used to clarify the nature of the interactions between guest (citronella oil, citronellal or citronellol) and host (β-cyclodextrin) was DSC. It is generally

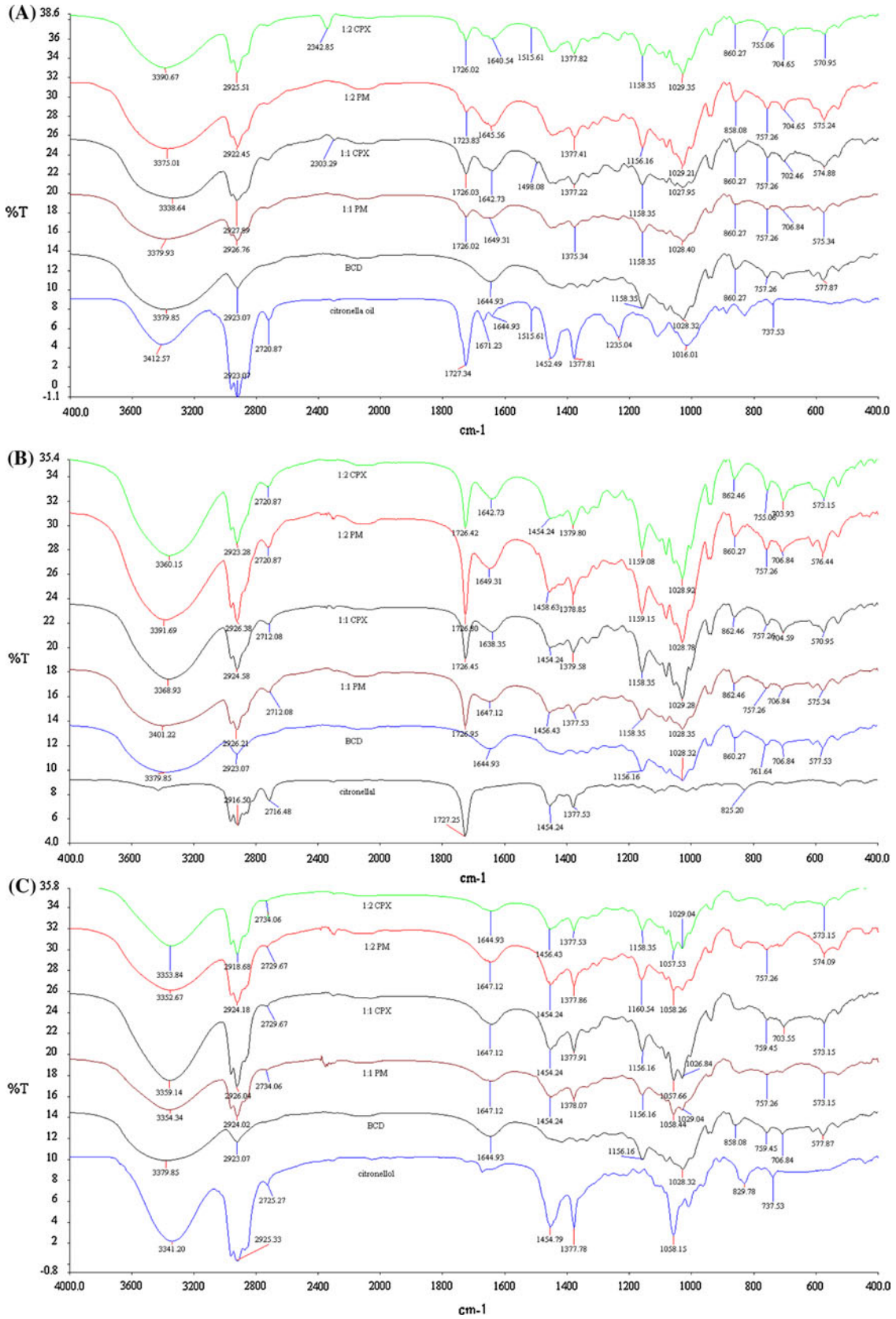


Fig. 3 FTIR spectra of pure compound, β-cyclodextrin (BCD), inclusion complexes at weight ratios 1:1 (1:1 CPX) and 1:2 (1:2 CPX) and physical mixtures at weight ratios 1:1 (1:1 PM) and 1:2 (1:2 PM); citronella oil (**a**), citronellal (**b**) and citronellol (**c**)

Table 4 The assignments of the most intense vibration bands of β-cyclodextrin

Vibration mode					
O–H stretch (cm^{-1})	C–H stretch (cm^{-1})	O–H bend (cm^{-1})	C–O stretch (cm^{-1})	CO/CC stretch (cm^{-1})	Pyranose ring vibration (cm^{-1})
3380	2923	1645	1156	1028	762

recognized that the inclusion of the guest into the cyclodextrin cavity results in changes to the microscopic property of the guest [23].

The thermal curves of pure citronella oil, β-cyclodextrin, inclusion complexes and their corresponding physical mixtures are shown in Fig. 4a. A sharp peak of citronella oil and a broad peak of β-cyclodextrin appeared at 121 and 124 °C, respectively. The peak position at 124 °C was due to the evaporation of water molecules from the β-cyclodextrin [24]. The inclusion complexes, 1:1 CPX and 1:2 CPX, showed one small endothermic peak at different positions from the pure citronella oil. The absence of the endotherm of citronella oil at 121 °C indicates a formation of the inclusion complexes [25–27]. Instead of showing two endothermic peaks, one for citronella oil (at 121 °C) and one for β-cyclodextrin (at 124 °C), the physical mixtures, 1:1 PM and 1:2 PM, exhibited one endothermic peak at around 147 °C.

As seen in Fig. 4b, DSC thermograms of pure citronellal and β-cyclodextrin exhibited peaks at 144 and 124 °C, respectively. The peak positions of the inclusion complexes (1:1 CPX, 1:2 CPX) were shifted to 136 °C, whereas the peaks of the physical mixtures (1:1 PM, 1:2 PM) were positioned at 144 °C, which was identical to that of pure citronellal. It was noted that the endothermic peaks of the physical mixtures were quite broad and large. In addition, there were no endothermic peaks that corresponded to that of β-cyclodextrin. The endothermic peak of citronellal in the inclusion complexes, 1:1 CPX and 1:2 CPX, was absent, indicating the formation of the inclusion complexes between citronellal and β-cyclodextrin [25–27].

Figure 4c demonstrates DSC curves of pure citronellol, pure β-cyclodextrin, inclusion complexes and physical mixtures. The broad peak of citronellol was observed at 151 °C. The endothermic peaks for 1:1 CPX, 1:2 CPX, 1:1 PM and 1:2 PM were shifted to 121, 126, 147 and 147 °C, respectively. The peak areas of the physical mixtures were much higher than those of the inclusion complexes. Unlike 1: 1 CPX, the formation of the inclusion complex was clearly confirmed for 1:2 CPX, since the endothermic peak of citronellol was entirely absent. In general, there were some differences in peak positions and peak areas of the pure compounds, the inclusion complexes and the corresponding physical mixtures.

The combined use of different characterization techniques has provided practical evidence for formation of complexes. In the current study, the formation of the inclusion complexes was clearly proven by the SEM technique. In the case of the FTIR method, the preparation of the samples for IR measurement, especially grinding and compressing the samples, may change the microstructures of the test compounds, in particular physical mixtures. Therefore, there were no clear differences in the FTIR spectra between the inclusion complexes and their corresponding physical mixtures. Theoretically, the IR spectra of physical mixtures should be similar to the synthetic spectra producing by the addition of active substance and β-cyclodextrin. For DSC analysis, the high heating used in the DSC technique may affect the thermal behavior of the physical mixtures and thus result in the shift of their endothermic peaks when compared with the pure compounds. Other characterization methods such as nuclear magnetic resonance spectroscopy (NMR) may be required to further investigate the interactions. Nevertheless, the obtained data offer us some fundamental information on the possibility of developing mosquito repellent formulations in our subsequent research.

Evaporation of the inclusion complexes of citronella oil

The evaporation of citronella oil and its inclusion complex products, 1:1 CPX and 1:2 CPX, determined at 120, 90 and 50 °C is illustrated in Fig. 5. It was clearly demonstrated that at 120 °C the inclusion complexes markedly reduced the amount of oil loss in comparison with normal citronella oil. The percent unevaporated oil in the inclusion complex at weight ratio of 1:2 (1:2 CPX) was statistically higher than that in the inclusion complex at weight ratio of 1:1 (1:1 CPX) (Student's t test, $p < 0.05$). At 50 min, the rank order of percent unevaporated oil was found to be 1:2 CPX > 1:1 CPX > citronella oil (normal). These results indicate that β-cyclodextrin could diminish the volatility of citronella oil. Similar results were observed at 90 °C, although the significant differences in percent unevaporated oil began at 10 min of experimental period. At 50 °C, there were no marked differences in the percent unevaporated oil in the three samples. Notably, the differences in the percentage of unevaporated oil in the three products decreased when the test temperatures were reduced to 90 and 50 °C.

Citronella oil formulations

All prepared formulations of citronella oil, F2, F3, and F4, showed good appearances with distinctive citrus scent. The strongest odor was noticed with the formulation F2 containing normal citronella oil. The formulations were

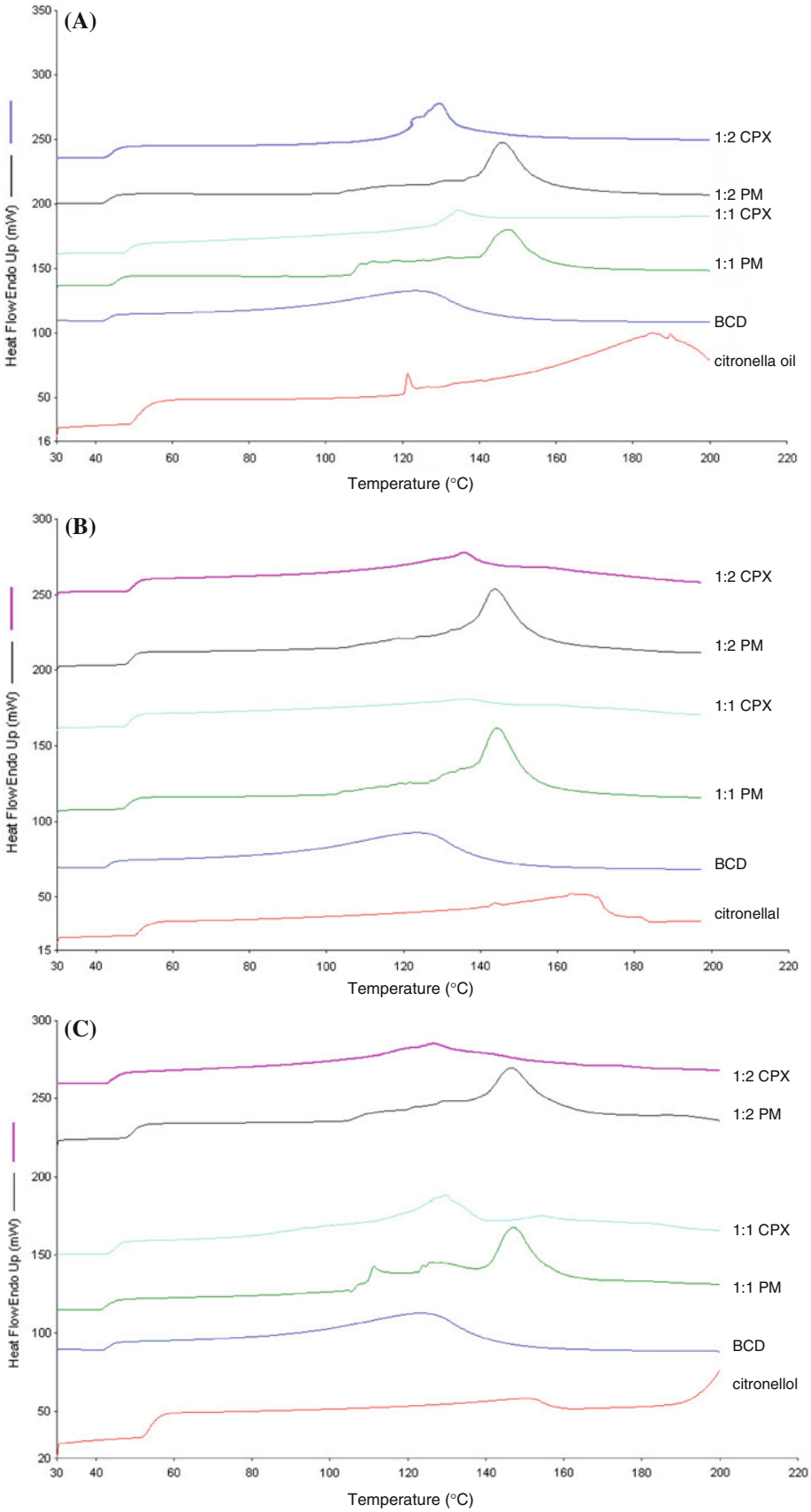


Fig. 4 DSC thermograms of pure compound, β-cyclodextrin (BCD), inclusion complexes at weight ratios 1:1 (1:1 CPX) and 1:2 (1:2 CPX) and physical mixtures at weight ratios 1:1 (1:1 PM) and 1:2 (1:2 PM); citronella oil (**a**), citronellal (**b**) and citronellol (**c**)

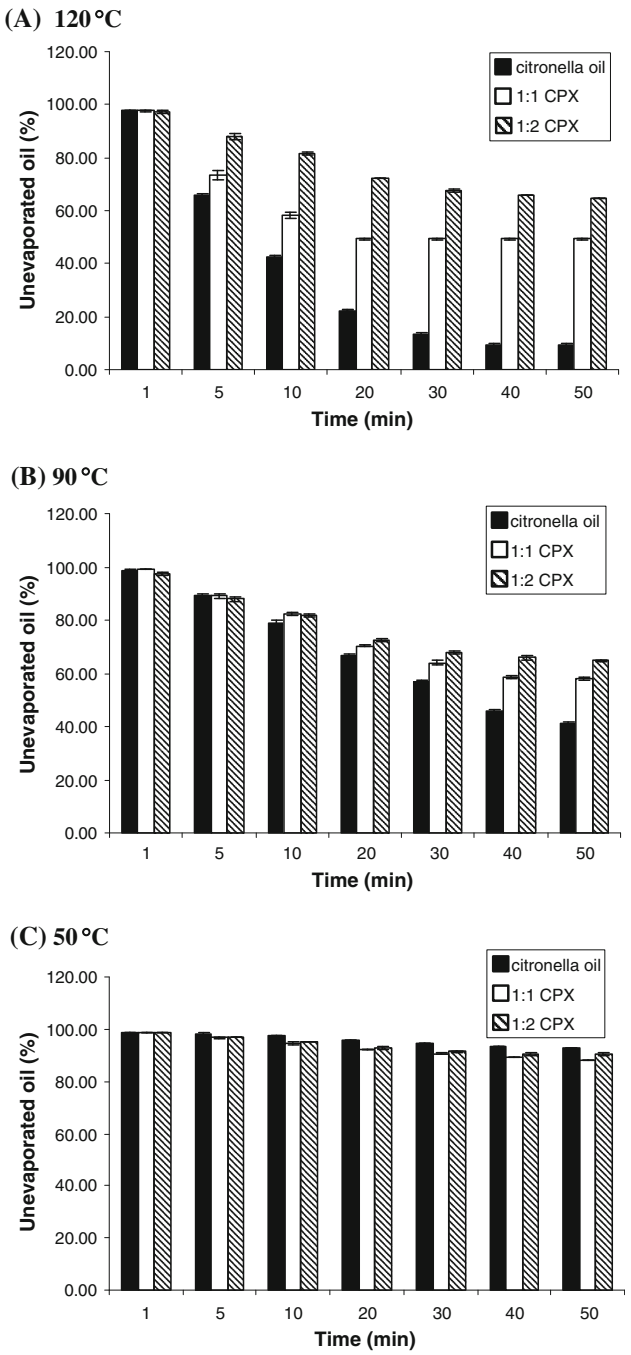


Fig. 5 Unevaporated citronella oil at three different temperatures; 120 (**a**), 90 (**b**) and 50 °C (**c**). Each point represents mean ± SD ($n = 3$, where n is number of samples)

homogeneous and had smooth textures. The textures of formulations F2 and F3 were smoother than that of the formulation F4. Formulation F4 appeared cream-like due to high amount of β-cyclodextrin in the formulation. The color of formulation F2 appeared yellow-white, while the formulations F3 and F4, which contained 1:1 CPX and 1:2 CPX, respectively had white color. The pH values of the formulations F2, F3 and F4 in comparison with the pH of the commercial product F1 are summarized in Table 5. The pH values of the formulations were in the ranges of 4.8–5.5. It should be pointed out that the pH of all formulations was weakly acidic, and thereby should be suitable for skin application. The rank order of pH values was found to be F4 > F1 > F2 > F3.

It is seen from Table 5 that the viscosities of the formulations were varied with the compositions of the formulations. Formulation F1, which was the solution-type lotion, exhibited the lowest viscosity, while formulation F4, which contained the inclusion complex (1:2 CPX), showed the highest viscosity (ANOVA, $p < 0.05$). In the case of the formulations F3 and F4, the relative high viscosities were due to the presence of β-cyclodextrin. Practically, the high viscosities of these formulations may cause some difficulties when they are applied onto the skin surface.

In vitro release of citronella oil

As seen in Fig. 6, the amount of citronella oil released from the commercial product F1 (solution type-lotion) was much lower than those from the prepared formulations F2, F3 and F4 at every sampling period (1–24 h). For the first 2 h, the amount of citronella oil released from the formulation F2 was similar to that from formulations F3 and F4, which contained the inclusion complexes. However, from 3 to 10 h, it was observed that the amount of citronella oil released from the formulation F2 was higher than that from the inclusion complex loaded formulations F3 and F4. At 10 h, the rank order of cumulative amounts of citronella oil released appeared to be F2 > F3 > F4 > F1 (see Table 5). At this sampling time, the cumulative release of citronella oil from the formulation F2 was significantly higher than that of the formulations F3 and F4. Although the amount of citronella oil released from the formulation F3 (1:1 CPX) seemed to be higher than that of the formulation F4 (1:2 CPX), there were no significant differences in the amounts of citronella oil released from these two formulations.

Table 5 shows the release rates and the amount of citronella oil released at 10 h of experimental period. The release rates of each formulation were obtained from the slopes of the release curves during 1–10 h (linear portion). The results showed the values of the coefficient of determination (R^2) to be over 0.9 for all formulations. The release rate of citronella oil from formulation F1 was significantly lower than that from the other formulations, F2, F3 and F4 (one-way ANOVA, $p < 0.05$). The release rate of citronella oil from formulation F2 (normal citronella oil) was statistically higher than that from the formulations F3 and F4 which contained the inclusion complexes, indicating that the inclusion complexes could retard the release of citronella oil. Nevertheless, there were no significant

Table 5 pH, viscosity and release parameters of citronella oil lotions (mean ± SD, $n = 3$)

Formulation	pH	Viscosity (cp)	Release rate (mg/cm^2/h)	R^2	Cumulative amount of citronella oil released at 10 h (mg/cm^2)
F1	5.12 ± 0.02	2.96 ± 0.07[a] (shear rate 85 s^{-1})	0.3663 ± 0.0343	0.9833	5.0750 ± 0.4496
F2	4.97 ± 0.04	77.14 ± 1.56[a] (shear rate 85 s^{-1})	0.7463 ± 0.0050	0.9197	10.3659 ± 0.1058
F3	4.78 ± 0.03	1,054.18 ± 45.47[a] (shear rate 8.5 s^{-1})	0.6532 ± 0.0233	0.9445	9.3467 ± 0.1388
F4	5.48 ± 0.04	56,188.01 ± 138.54[b] (speed 2.0 rpm)	0.5705 ± 0.0314	0.9372	8.7431 ± 0.1132

F1 commercial product, *F2* prepared lotion containing citronella oil, *F3* prepared lotion containing 1:1 CPX, *F4* prepared lotion containing 1:2 CPX

[a] Spindle SC4-31 was used to measure apparent viscosity

[b] Spindle LV3 was used to measure apparent viscosity

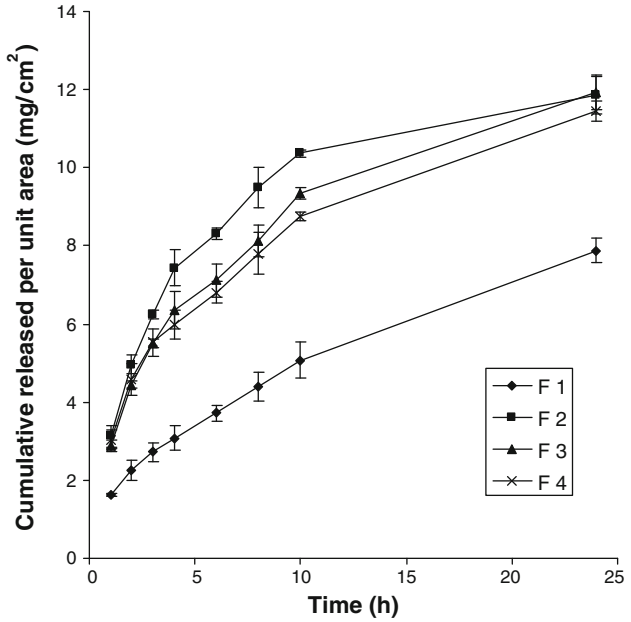


Fig. 6 In vitro release profiles of citronella oil from different formulations during 24 h. Each point represents mean ± SD ($n = 3$). *F1* commercial product, *F2* prepared lotion containing citronella oil, *F3* prepared lotion containing 1:1 CPX, *F4* prepared lotion containing 1:2 CPX

differences in the release rates of citronella oil from the inclusion complexes at weight ratios of 1:1 and 1:2. The present data suggest that further increase of the weight ratio of β-cyclodextrin is not practically useful.

Relationship between viscosity and release rate of citronella oil

For the prepared formulations F2, F3 and F4, the viscosity of the formulation appeared to affect the release rate of citronella oil. Formulation F2, which possessed the lowest viscosity, provided the highest release rate and vice versa. The influence of viscosity on the release of drugs has been previously reported [28].

Mosquito repellent activity of lotions

The mosquito repellency of the prepared lotions and the commercial product of citronella oil is summarized in Table 6. The rank order of the protection time was F3 > F2 = F1 > F4. The longest protection time was observed in formulation F3, containing the inclusion complex at weight ratio of 1:1, indicating that β-cyclodextrin could prolong the insect repellent activity of citronella oil. However, this was not the case for the inclusion complex at weight ratio of 1:2. This is probably due to the fact that citronella oil is strongly trapped in β-cyclodextrin structure. Therefore, it could not adequately release and create a vapor barrier deterring mosquitoes in a short period of time. Furthermore, the short protection time of formulation F4 could be attributed to the high viscosity of the formulation. For citronellal and citronellol, the inclusion complex products did not prolong the protection time when compared with the normal citronellal or citronellol. In addition to citronella oil, the lotions of its major components, citronellal and citronellol, both normal and the inclusion complexes (1:1), were also prepared and tested for their mosquito repellency. In the current study, the concentration of the terpene used was 10% w/w. In the case of citronellal, the protection time of the lotion containing normal citronellal was the same as that containing the complex product. A decrease in the protection time was observed for the inclusion complex of citronellol. Interestingly, the protection time of normal citronellol (F7)

Table 6 Mosquito protection times of the prepared lotions and the commercial product (mean ± SD, $n = 3$ where n is the number of human volunteers)

Formulation	Average protection time (h)	Protection time of three volunteers (h)		
		No.1	No.2	No.3
F1	1.3 ± 0.3	1.5	1.5	1.0
F2	1.3 ± 0.3	1.0	1.5	1.5
F3	1.8 ± 0.3	1.5	2.0	2.0
F4	0.7 ± 0.8	1.5	0.5	0.0
F5	1.3 ± 0.3	1.5	1	1.5
F6	1.3 ± 0.3	1.5	1	1.5
F7	1.7 ± 0.8	1.5	1	2.5
F8	1.2 ± 0.3	1	1	1.5

F1 commercial product, *F2* prepared lotion containing citronella oil, *F3* prepared lotion containing 1:1 CPX citronella oil, *F4* prepared lotion containing 1:2 CPX citronella oil, *F5* prepared lotion containing citronellal, *F6* prepared lotion containing 1:1 CPX citronellal, *F7* prepared lotion containing citronellol, *F8* prepared lotion containing 1:1 CPX citronellol

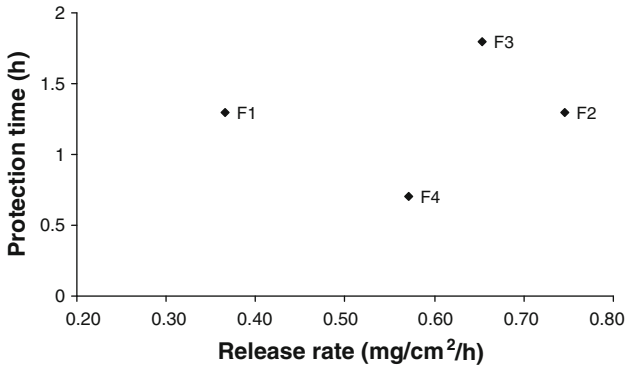


Fig. 7 Relationship between release rate of citronella oil from the formulations F1, F2, F3 and F4 and their protection time against *A. aegypti*. *F1* commercial product, *F2* prepared lotion containing citronella oil, *F3* prepared lotion containing 1:1 CPX, *F4* prepared lotion containing 1:2 CPX

seemed to be longer than that of normal citronella oil (F2) and citronellal (F5).

In the current study, all preparations did not meet the requirement of the National Institute of Health, Thailand because their protection times against *A. aegypti* were less than 2.0 h. Nevertheless, reapplications of these preparations on a frequency basis should compensate for the short duration of action of these citronella oil-based mosquito repellents. Indeed, repeated applications at certain time intervals are generally indicated on the labels of the commercial botanical repellents [7].

Relationship between release rate and protection time

As seen from Fig. 7, there was no linear correlation between the release rates and the protection times of the formulations, suggesting no simple relationship between these two parameters. In addition, a linear relationship did not exist between the percentage of unevaporated oil and the protection time of the prepared lotions (data not shown).

Conclusions

In many tropical areas, like Thailand, using mosquito repellents is one of the popular methods to repel or deter mosquitoes from biting humans. Citronella oil, a volatile oil which possesses mosquito repellent activity, was selected to form inclusion complexes with β-cyclodextrin in an attempt to reduce its volatility and accordingly enhance its repellent efficacy. Based on the current investigation, it appeared that the promising citronella oil-based repellent lotion could be obtained with citronella oil–β-cyclodextrin inclusion complex at weight ratio of 1:1 (F3). In vitro release study revealed that the release rate of citronella oil from the formulation F3 was statistically lower than that from the prepared lotion containing normal citronella oil (F2). These results suggest a controlled release of the citronella oil from the inclusion complex (1:1). The efficacy of this formulation tested by the human-bait technique showed that the protection time of this lotion was less than 2 h, which did not meet the criteria of the National Institute of Health, Thailand. Nevertheless, the protection time of the formulation F3 was longer than that of the commercial product.

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