Original Article

DOI: 10.5582/ddt.2011.v5.5.246

Development of microemulsion of a potent anti-tyrosinase essential oil of an edible plant

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ABSTRACT: The aim of this study is to develop a microemulsion product from a plant essential oil having the highest anti-tyrosinase activity. The *in vitro* anti-tyrosinase activity of six essential oils extracted from six edible plants was compared. The oil of *Cymbopogon citratus* demonstrated the highest activity which was significantly nontoxic to normal human cells. The GC-MS data indicated that geranial and neral are the major compounds in the oil. The phase diagram composed of *C. citratus* oil, water, and surfactant mixture was conducted by a titration method. Ethyl alcohol was found to be the most suitable cosurfactant for the *C. citratus* oil microemulsion. The results revealed that the amount of oil and water played an important role in microemulsion conductivity and type. The most desirable o/w type of *C. citratus* oil microemulsion was found to be composed of 20% oil, 30% water, and 50% surfactant mixture of a 2:1 weight ratio of Tween 20 and ethyl alcohol.

Keywords: *Cymbopogon citratus*, antityrosinase, essential oil, microemulsion, plant

1. Introduction

Tyrosinase is an enzyme involved in melanin production *via* an enzymatic oxidative pathway which is of considerable importance in the coloring of skin, hair, eyes, and in some food browning (*1*,*2*). Chemical agents that demonstrate anti-tyrosinase activity have been used in clinical medicine for the treatment of some dermatologic disorders associated with melanin hyperpigmentation (*3*), and are useful in cosmetic preparations and the food industry (*4*,*5*). Melanin production might be responsible for some of the histo-pathological features exclusive to malignant cancer. Therefore, anti-tyrosinase substances may be clinically helpful in dealing with skin cancer. In recent years, more attention has been paid to the use of natural products instead of chemical or synthetic compounds in order to be not only economical but also environmentally friendly as well as safe. Moreover, the exploration of green technology and of low cost raw materials is an important feature for the industry as well as for making improved use of plant resources. Recently, the anti-tyrosinase activity from the methanol and acetone extracts of certain plants has been reported (*6*). However, there is less data from essential oils of natural plants. Therefore, searching for plant essential oils which possesses high anti-tyrosinase activity is of interest. The attempt is to develop a suitable microemulsion from such oils would be beneficial for pharmaceutical and cosmetic applications.

A microemulsion is a thermodynamically stable and clear dispersion composed of two immiscible liquids, usually oil and water. Microemulsions have received increasing attention as drug delivery systems during the past several years because they have many potential characteristics suitable for pharmaceutical and cosmetic applications such as enhanced aqueous solubilization of lipophilic compounds, increased drug permeation rates, good thermodynamic stability, and ease of preparation (*7*,*8*). The immiscible lipophilic and hydrophilic liquids in microemulsions can be assembled into a one clear liquid phase system by using surfactant and cosurfactant (*9*,*10*). In most cases, the lipophilic liquids used for microemulsion are inactive or less biologically active oils. Research on plants have explored the potential of essential oils extracted from plants for anti-fertility (*11*,*12*), anti-antioxidant (*13*,*14*), anti-inflammatory, and antimicrobial activities (*15*-*17*). Recently the anti-tyrosinase activity of rose, carnation, and hyacinth oils has been reported (*18*).

In the present study, several edible essence plants believed by local people in Thailand to support skin whitening were collected. The anti-tyrosinase

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activity of the essential oils extracted from these plants was compared. The microemulsion of the oil which possessed the highest activity was developed by using a phase diagram. The effect of oil, water, and surfactant quantity as well as the type of cosurfactant on the characteristics of the microemulsion was examined.

2. Materials and Methods

2.1. *Plant materials and essential oil extraction*

Six edible plant samples; *Cymbopogon citratus*, *Eryngium foetidum*, *Ocimum canum*, *Alpinia galanga*, *Curcuma longa*, and *Curcuma zedoaria* were collected from a local garden located in Chiang Mai, Thailand during June 2010. The plants were authenticated and the voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The fresh aerial parts of *C. citratus*, *E. foetidum*, and *O. canum* and the fresh rhizomes of *A. galanga*, *C. longa*, and *C. zedoaria* were cut into small pieces and subjected to a hydro-distillation apparatus for 3 h using a Clevenger type apparatus to collect the oil. The essential oils were dried over anhydrous sodium sulfate and kept in light protected containers at 4ºC until further experiments.

2.2. *Chemicals and reagents*

Mushroom tyrosinase and L-dopa were purchased from Fluka Chemical Co. (Japan). Ethyl alcohol, propyl alcohol, and butyl alcohol were obtained from Fisher Chemicals (Loughborough, UK). Dimethylsulfoxide (DMSO) was from Fisher Scientific (Leicestershire, UK). These reagents were of analytical grade. Polyoxyethylene sorbitan monolaurate (Tween 20) of pharmaceutical grade was purchased from Namsiang Co., Ltd. (Bangkok, Thailand). Other chemicals were of the highest grade available.

2.3. *Determination of antityrosinase activity*

Anti-tyrosinase activity of the essential oils was determined using the modified dopachrome method with L-dopa as substrate (*19*). Assays were conducted in a 96-well microtitre plate. Test samples were dissolved in 50% DMSO. Each well contained 40 µL of sample, 80 µL of phosphate buffer solution (PBS) (0.1 M, pH 6.8), 40 µL of tyrosinase (200 units/mL) and 40 µL of L-dopa (2 µM). The microplate reader was read at 450 nm. Each sample was accompanied by a blank that had all the components except L-dopa. Results were compared to a control consisting of 50% DMSO in place of the sample. The anti-tyrosinase activity of the oil samples expressed as percentage tyrosinase inhibition was calculated using the following equation.

$$\% \text{ Inhibition} = 100 \times (Ac - As)/Ac$$

where Ac is the absorbance of control and As is the absorbance of sample.

2.4. *Cytotoxicity tests*

The cytotoxicity of oil samples on normal human cells using peripheral blood mononuclear cells (PBMCs) was determined using a colorimetric technique and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PBMCs were plated in 96-well plates to obtain a cell concentration of 1×10^5 cells/well. Serial dilutions of oils were added to the wells. The wells were incubated in a 37°C, 5% CO_2 and 90% humidity incubator for 48 h. After the corresponding time, 15 µL of MTT at 5 mg/mL was added into each well in the 96-well plate and further incubated for 4 h in a 37°C, 5% CO_2 and 90% humidity incubator. One hundred and seventy microlitres of medium with MTT was removed from every well and 100 µL DMSO was added to each well to extract and solubilize the formazan crystals by incubating for 20 min in a 37°C, 5% CO_2 incubator. Finally, the plate was read at 540 nm using an ELISA Reader. The percentage of cell viability was calculated by the following formula.

$$\% \text{ Cell viability} = 100 \times (Ds - Dc)/Dc$$

where Ds is the OD of sample and Dc is the OD of control.

2.5. *GC-MS analysis*

The essential oil which showed the highest anti-tyrosinase activity was subjected to GC-MS in order to analyze the components existing in the oil. The GC-MS analysis was performed on an Agilent 6890 gas chromatograph coupled to electron impact (EI, 70 eV) using an HP 5973 mass selective detector fitted with a fused silica capillary column (HP-5MS) supplied by HP, Palo Alto, CA, USA (30.0 m × 250 mm, *i.d.* 0.25 mm film thickness). The analytical conditions were; carrier gas: helium (ca. 1.0 mL/min), injector temperature: 260°C, oven temperature: 3 min isothermal at 100°C (No peaks before 100°C after first injection), then at 3ºC/min to 188°C and then at 20°C/min to 280°C (3 min isothermal), and detector temperature: 280°C. The programmed-temperature Kováts retention indices (RI) were obtained by GC-MS analysis of an aliquot of the volatile oil spiked with an *n*-alkane mixture containing each homologue from *n*-C11 to *n*-C27. Identification of the compounds was based on a comparison of their mass spectra database (WILEY&NIST) and spectroscopic data. The percentage amount of each component was calculated based on the total area of all peaks

obtained from the oil. The data obtained were used as a standard for further batches of the oil.

2.6. *Construction of phase diagrams*

The pseudo-ternary phase diagram of *C. citratus* oil was constructed using a water titration method (*20*). Tween 20 was mixed with different cosurfactants (ethyl alcohol, propyl alcohol, or butyl alcohol) at a weight ratio of 2:1 to obtain a surfactant mixture. For each phase diagram, the weight ratios of the oil and the surfactant mixture were varied as ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. These mixtures were titrated with water, under moderate agitation at ambient temperature. The phase boundary was determined by observing the changes of the sample appearance going from transparent to turbid. The experiment was done in triplicate. The pseudo-ternary phase diagram was drawn by SigmaPlot for Windows version 10.0. The samples were classified as microemulsions when they appeared as a clear liquid.

2.7. *Preparation and characterization of microemulsions*

Several microemulsion formulas of *C. citratus* oil were developed by mixing the oil with other components presented in the microemulsion region of the most suitable phase diagram obtained with moderate agitation at ambient temperature. The microemulsions obtained were characterized for conductivity and microemulsion type.

2.8. *Conductivity measurement*

The electrical conductivity of the microemulsion was measured using a Cyberscan CON 11: hand-held conductivity meter (Eutech instruments, Singapore) using a conductivity/TDS electrode cell. The experiment was performed at 25 ± 1.0°C by dipping the electrode into the test sample until equilibrium was reached and the reading became stable. The performance was done in triplicate.

2.9. *Microemulsion type determination*

The type of microemulsion was judged by color diffusion using oil soluble sudan red and water soluble methylene blue solutions. If the red diffused faster than the blue, the microemulsion was the w/o type. On the contrary, if the blue diffused faster than red, it was the o/w type.

3. Results and Discussion

3.1. *Antityrosinase activity and safety of the oils*

It is known that tyrosinase, a copper-containing monooxygenase, widely distributed in microorganisms, animals, and plants, is an important enzyme implicated in melanin biosynthesis mainly using two distinct reactions of monophenolase and diphenolase activities (*21*,*22*). The overall activity of this enzyme can cause epidermal hyperpigmentation which leads to various dermatological disorders such as melasma, freckles, age spots, and skin cancer (*23*). Compounds having an anti-tyrosinase activity hence are, so far, the agents of interest in treatment and prevention of those dermatological disorders. The essential oils extracted by hydro-distillation of six different edible plants used in this study demonstrated their activity on inhibition of tyrosinase activity as shown in Figure 1. Among them, the oil of *C. citratus* displayed the strongest anti-tyrosinase activity with an IC_{50} of 0.5 mg/mL followed distantly by *C. longa*, and *A. galanga* oils with an IC_{50} of 3.6 mg/mL as shown in Table 1. Compared to the other plant extracts previously reported (*19*) *e.g. Etlingera littorali*, *E. rubrostriata*, *E. maingayi*, and *E. fulgens*, the essential oil of *C. citratus* was more effective in tyrosinase inhibition than those plants. The essential oils of *C. citratus*, *C. longa*, and *A. galanga* hence were considered to be a group of high activity whereas the other three plant oils of *O. canum*, *E. foetidum*, and *C. zedoaria* in which the IC_{50} was higher were considered to be a low activity group. The three oils of the high potential group then were selected for further tests of cytotoxicity in order to interpret the safety to normal human cells.

The cell viability of human peripheral blood mononuclear cells (PBMCs) could indicate the safety of the oil samples. In general, cell viability greater than 80% after exposure to test samples is recognized as safe for human use (*24*). Figure 2 displays cell viability after contact with different concentration of the oils. The results demonstrate that after 48 h incubation with *C. citratus*, *C. longa*, and *A. galanga* oils, the cell viability was more than 80%. More importantly, it is noted that the cell viability after *C. citratus* oil exposure was constantly near 100% whereas that of *C. longa* and *A. galanga* oils was slightly decreased with higher oil concentration. This result indicates *C. citratus* oil is nontoxic to human cells.

According to the extremely highest anti-tyrosinase activity of *C. citratus* oil as well as its complete safety to human cells, this oil was considered to be the most effective in the inhibition of melanin formation and is suitable for further study. Prior to microemulsion development, the chemical composition of the oil was investigated by GC-MS. The result is demonstrated in Table 2. Twelve volatiles were identified, making up 89.5% of the total composition. The majority of this oil was monoterpene which comprised up to 85.2%. The most abundant constituents were geranial (42.0%) and neral (32.1%). This finding was in accordance with the previous published data on *C. citratus* oils by

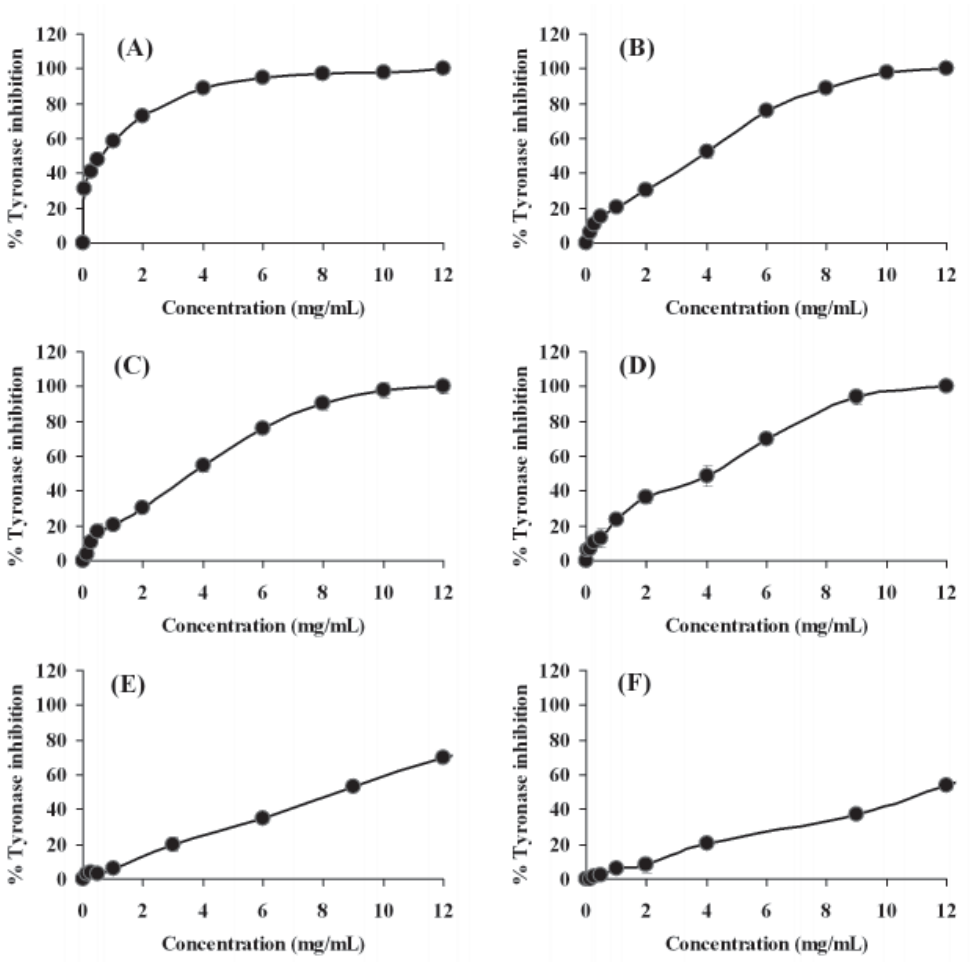

Figure 1. Effect of different oil concentrations of *C. citratus* (A), *C. longa* (B), *A. galanga* (C), *O. canum* (D), *E. foetidum* (E), and *C. zedoaria* (F) on inhibition of tyrosinase activity.

Table 1. The IC_{50} of anti-tyrosinase activity of the essential oils

Scientific name	IC_{50} (mg/mL)
C. citratus	0.5
C. longa	3.6
A. galanga	3.6
O. canum	4.2
E. foetidum	8.2
C. zedoaria	10.2

Blanco *et al*. (*25*) that geranial and neral were the main compounds of this oil. It is known that geranial and neral are isomers of citral. Matsuura *et al*. (*26*) reported that the tyrosinase inhibitory activity of citrus essential oils was relative to the abundance of citral. Our result was similar to this report that geranial and neral were related to this activity. Therefore, it was considered that these two compounds might play an important role in anti-tyrosinase activity.

3.2. *Phase diagram of microemulsion*

The pseudo-ternary phase diagrams with three different cosurfactants (C2-C4 alcohols) are shown in Figure 3. The transparent microemulsion region (ME) is

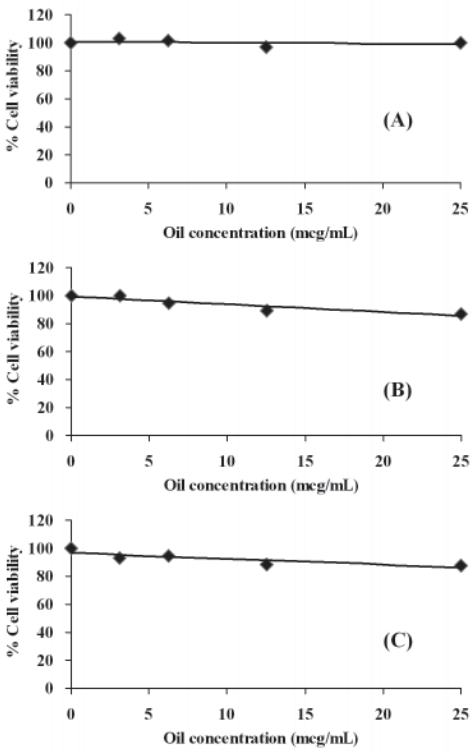

Figure 2. Cytotoxicity of the essential oils of *C. citratus* (A), *C. longa* (B), and *A. galanga* (C), on normal human PBMCs.

presented in the phase diagrams. It is obviously seen that alcohols of C2-C4 had a different effect. This is in contrast to the findings reported by Alany *et al*. (*27*) where no distinct trends were observed in the homologous series of the alcohols. However, the effect of alcohols may be influenced by the oil and surfactant combination used in the pseudoternary system. Among the three alcohols used in the present study, C2 alcohol (ethyl alcohol) showed the largest ME. Ethyl alcohol is safe and widely used in skin products. Moreover, it was reported that ethyl alcohol could act as a skin enhancer (*28*). Hence, ethyl alcohol was considered to be the most suitable cosurfactant for the *C. citratus* oil microemulsion. Therefore, it was selected to be used as a cosurfactant for the further study.

Table 2. Chemical compositions of the essential oil of *C. citratus*

Retention time (min)	Component	Peak area (%)
3.48	*trans*-β-Ocimene	0.58
4.07	*n*-Undecene	1.4
5.32	D-Camphor	0.49
5.75	(+)-Borneol	0.35
7.25	Neral	32.07
7.55	Geraniol	5.21
8.13	Geranial	42.01
11.38	Piperitenone oxide	2.25
11.53	Decaoic acid	0.79
11.72	α-Copaene	1.4
20.66	β-Maaliene	0.94
21.02	α-Cadinol	2.04
	Total	89.53

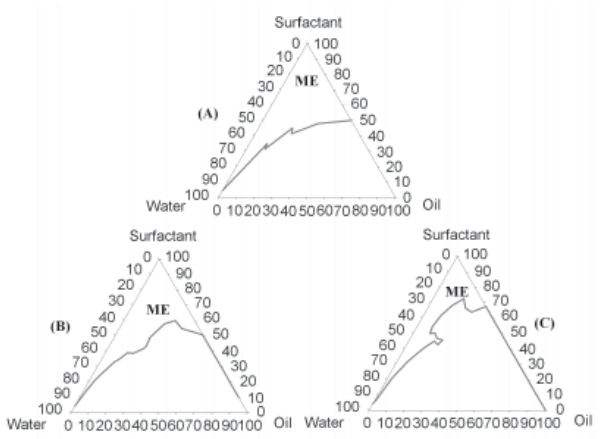

Figure 3. Pseudo-ternary phase diagrams of *C. citratus* oil microemulsions (ME) with different cosurfactants; ethyl alcohol (A), propyl alcohol (B), and butyl alcohol (C).

3.3. *Preparation and characterization of microemulsions*

In this study, three criteria were defined for the selection of microemulsion formulations from the developed phase diagrams; *i*) the percentage of oil should be more than 10%, *ii*) the type of microemulsion should be o/w, and *iii*) the amount of surfactant should be minimized. Because the ME in the pseudo-ternary phase diagram of *C. citratus* oil could be obtained using different amounts of oil, water, and surfactant in the system, eight formula compositions shown in Table 3 were prepared. The results revealed that all microemulsions obtained were clear with pale yellowish color solutions. The conductivity of each formulation was performed. It was found that high conductivity was obtained when the percentage of water was increased or when the oil was decreased. The dye solubility test is an excellent tool for determination of microemulsion type. In this study, the oil soluble red dye was not miscible with the o/w type but completely miscible with the w/o type whereas the water soluble blue dye demonstrated complete miscibility with microemulsions of the o/w type but not with the w/o type. The results showed that the o/w type could be obtained from the high conductivity microemulsion whereas in low conductivity microemulsions, the w/o type was observed. The result also indicated that when the conductivity of the system was above 40 µS/cm, the type of *C. citratus* oil microemulsion was reversed from w/o to o/w. Therefore, this conductivity value was considered to be a critical point of the microemulsion type. This value is slightly lower than previously reported by Yuan, *et al*. (*29*). However, this finding was in agreement with other authors that increasing the water fraction affected higher conductivity and o/w microemulsions were obtained (*30*). The present results

Table 3. The conductivity and emulsion type of various formulas of *C. citratus* oil microemulsions

Formulation	Composition (w/w)			Conductivity (µS)	Dye solubility test*
	Oil	Surfactant mixture	Water		
ME-1	10	50	40	98.47 ± 0.15	A
ME-2	10	60	30	68.17 ± 0.06	A
ME-3	10	70	20	45.33 ± 0.06	A
ME-4	20	50	30	63.50 ± 0.10	A
ME-5	20	60	20	41.37 ± 0.15	A
ME-6	30	50	20	32.73 ± 0.06	B
ME-7	30	60	10	26.30 ± 0.00	B
ME-8	40	50	10	19.08 ± 0.04	B

* A, Miscible with water soluble dye = o/w type; B, Miscible with oil soluble dye = w/o type.

demonstrate that the increase in percentage of oil could lead the microemulsion type to be w/o. The results from Table 3 exhibited that the highest percentage of oil that could produce a o/w type microemulsion could be obtained from formula ME-4 and ME-5 with an oil content of 20%. However, ME-4 contained less surfactant that ME-5. Therefore, formula ME-4 was considered to be the most desirable microemulsion of *C. citratus* oil.

4. Conclusion

The essential oil of *C. citratus* demonstrated the highest anti-tyrosinase activity among six edible plant oils. The GC-MS indicated that geranial and neral are the major compounds in the oil. The safety data suggested that *C. citratus* oil can be a promising plant oil effective and worthy for development of a microemulsion for anti-tyrosinase activity. The development of the *C. citratus* oil microemulsion indicated that ethyl alcohol was the best cosurfactant for the system using Tween 20 as a principle surfactant. The amount of oil and water in the formula played an important role on microemulsion conductivity and type. The most desirable formula for *C. citratus* oil microemulsion was composed of 20% oil, 30% water, and 50% surfactant mixture of a 2:1 weight ratio of Tween 20 and ethyl alcohol.

Acknowledgement

The authors are grateful for the financial support from the Thailand Research Fund (TRF) through grant number IUG 5080012. We also thank the Graduate school, Chiang Mai University for their support.

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(Received August 16, 2011; Revised October 13, 2011; Accepted October 14, 2011)