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Original Article

CHARACTERIZATION OF HYDRODISTILLATED POMELO PEEL OIL AND THE ENHANCEMENT OF BIOLOGICAL ACTIVITIES USING MICROEMULSION FORMULATIONS

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ABSTRACT

Objective: The present study aims to investigate the compositions and biological activities of essential oil extracted from pomelo peel and develop into microemulsions.

Methods: Four subspecies of pomelo including Kao-Namphung (KN), Kao-Puang (KP), Kao-Tang-Gwa (KT), and Kao-Yai (KY) were subjected to the hydrodistillation to yield essential oils. The constituents of each oil was analyzed by GC-MS. Radical scavenging activities were determined by ABTS and DPPH assays, whereas, lipid antioxidant activity was determined by linoleic acid peroxidation assay. Antityrosinase activity and safety on human PBMCs were also investigated. Pseudoternary phase diagrams were constructed to reveal the effects of each compostions on the microemulsion regions. The microemulsion was formulated and chracterized for the particle size, rheological behavior and biological activities.

Results: Limonene was the major constituent in KN, KP, KT, and KY oil which was detected up to 86.19%, 85.76%, 79.36%, and 80.20%, respectively. Among four oils, KT oil exhibited the highest radical scavenging, antioxidant and antityrosinase activities. The MTT assay revealed that KT oil had no toxicity on human PBMCs. The microemulsion formulation (ME) containing 15% KT, 36% Tween 20, 9% PEG 400, and 40% water, were formulated and chracterized. ME was transparent liquid with the particle size of 90.28 ± 1.60 nm. ME exhibited the Newtonian flow behavior with low viscosity (16.78 ± 0.12 Pas). In a comparison with KT oil, ME show significant higher radical scavenging and antioxidant activities (p< 0.01).

Conclusion: Development of microemulsion increased radical scavenging and antioxidant activities of KT oil and would be an attractive system for further development to effective topical products.

Keywords: Pomelo, Essential oil, Antioxidant, Antityrosinase, Cytotoxicity, Microemulsion, Pseudoternary phase diagram.

INTRODUCTION

Microemulsion is an isotropic colloidal system that is formed spontaneously from appropriate combinations of oil, water, and surfactant/co-surfactant mixtures [1]. These systems are currently of interest to the pharmaceutical scientist because of their considerable potential to act as drug delivery vehicles by incorporating a wide range of drug molecules [2]. Microemulsion is optically transparent since the internal phase droplet size ranges from 5 to 200 nm [3,4], which is below the wavelength of visible light. The key difference between microemulsions and emulsions is that microemulsions exhibit excellent thermodynamically stable whereas emulsions may exhibit excellent kinetic stabilitybut fundamentally thermodynamically unstable and will eventually phase separate [5]. In addition, the methods of preparation are distinctly different, since emulsions require a large input of energy while microemulsions do not need, leading to reduce the relative cost of commercial production [2]. As topical vehicles, microemulsions can increase the local or systemic delivery of compounds by enhancing their solubility, leading to greater amount of compounds incorporated in the microemulsion than other conventional topical formulations such as ointments, creams, gels, and lotions [6,7]. Moreover, the diffusional barrier of the skin may be modified depending on the composition of the microemulsion [8].

Many *Citrus* species have been widely utilized in foods, beverages and as fragrances in cosmetics [9] because of their excellent refreshing flavor of the essential oils as well as their sweet and delicious taste [10]. The center of origin and diversity of *Citrus* is generally considered to be Southeast Asia [11] since *Citrus* is grown in tropical and subtropical climates extending 40º north and south of the equator where winter temperatures are moderate [12]. One of the *Citrus* fruits that widely consumed is pomelo(*Citrus grandis* L.) which is monoembryonic species with the largest fruit among the *Citrus* genus [11]. In Thailand, there are many subspecies of pomelo cultivated such as Kao-Namphung, Kao-Puang, Kao-Tang-Gwa, Kao-Yai, etc. which are different in shape, color, size and taste. As people eat the endocarp, and the pulp has been used in the ancient time as antitoxic, appetize, cardiac stimulant and stomach tonic remedy, the fruit peel is a biomass waste [13]. In recent years, there are many studies investigate the pharmaceutical effects of the pomelo peel. The hexane extract shows antiproliferative effect on human leukaemia cells [14], while ethyl acetate extract from fruit tissues shows antioxidant effect [15]. Moreover, the fruit peel was used as an economical alternative substrate for fungal pectinase production [16]. Pomelo can give quantity of essential oil according to the oil glands which are commonly found in the stems, leaves, flowers, and fruits where they are positioned in the exocarp of the rind [17]. The active compounds found in the essential oil of pomelo fruit peel have strong ability in antimicrobial activity [13,18]. Many potential activities including antioxidant and antityrosinase activities of pomelo have been reported [19]. However, there is no knowledge on the effects of different subspecies. Therefore, this study aims to investigate the compositions, anti tyrosinase and antioxidant activities of the essential oil from various subspecies of pomelo in Thailand. The cytotoxicity of the oil was also evaluated to confirm its safety and topical microemulsion was then developed and characterized.

MATERIALS AND METHOD

Plant materials

Four subspecies of pomelo including Kao-Namphung (KN), Kao-Puang (KP), Kao-Tang-Gwa (KT), and Kao-Yai(KY) were purchased from local market in Chiang Mai, Thailand during February 2014. All plant samples were authenticated and voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand.

Chemical materials

ABTS(2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (1,1-Diphenyl-2-Picrylhydrazyl Radical), linoleic acid, tyrosinase

from mushroom, L- tyrosine, n-alkanes mixture containing each homologue from n-C8 to n-C20, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, Triton X-114, and propylene glycol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tween 20, Tween 80, and Span 80 were purchased from Acros Organics (New Jersey, USA). Glycerin, BP/USP was purchased from Malaysia. Polyethylene glycol 400, USP was purchased from Wilhelmshaven, Germany. Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). Ficoll-paque plus was purchased from Lymphoprep™Axis-Shield PoC AS, (Oslo, Norway). Disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium persulfate, ammonium thiocyanate, and ferrous chloride were purchased from Fisher Chemicals (Loughborough, UK). RPMI 1640, Penicillin, Streptomycin, and Trypan blue were purchased from GIBCO™ Invitrogen (Grand Island, NY, USA). Hydrochloric acid was AR grade purchased from Merck (Darmstadt, Germany). Ethanol, acetone, propan-2-ol, dimethyl sulfoxide (DMSO) were AR grade purchased from Labscan (Dublin, Ireland).

Distillation of essential oils

The fresh peel of pomelo was separately removed from the fruit, cut into small pieces and subjected to hydrodistillation for three hours using a Clevenger type apparatus. The essential oils obtained were stored in a refrigerator and protected from light until further use. Yields of each essential oil were calculated based on the weight of fresh exocarp. Density of each essential oil was analyzed by using pycnometer.

GC-MS analysis

The isolated essential oils were analyzed by GC-MS. The GC-MS analysis was performed on Agilent 6890 gas chromatography coupled to electron impact (EI, 70 eV) with HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) supplied by HP, 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 ºC, oven temperature: 3 min isothermal at 100 ºC (No peaks before 100 ºC after first injection), then at 3 ºC/min to 188 ºC and then at 20 ºC/min to 280 ºC (3 min isothermal), and detector temperature: 280 ºC. The programmed-temperature Kováts retention indices (RI) were obtained by GC-MS analysis of an aliquot of the volatile oil spiked with an n-alkanes mixture containing each homologue from n-C8 to n-C20. Identification of the compounds was based on a comparison of their mass spectra database (WILEY&NIST) and spectroscopic data[20].

Antioxidant activity

ABTS assay

Pomelo oils and microemulsion were test for ABTS radical cation scavenging activity using method reported by Fellegrin with slight modifications [21]. Briefly, ABTS solution (7 mM) was reacted with potassium persulfate (140 mM) solution and kept in the dark for 16 h to yield a dark colored solution containing $ABTS^{\cdot+}$ radical cation. Prior to use in the assay, the ABTS radical cation was diluted with ethanol for an initial absorbance of about 0.500 at 734 nm. After the addition of 1.0 mL of diluted $ABTS^{\cdot+}$ to 10 µL of sample, the absorbance was measured after 6 min of initial mixing. The percentage inhibition was calculated using the following equation; *% scavenging effect* = $[1 - (S / C)] \times 100$, when S is an absorbance of $ABTS^{\cdot+}$ with sample and C is an absorbance of $ABTS^{\cdot+}$ without sample. The experiment was done in triplicate.

DPPH assay

Pomelo oils and microemulsion were test for radical scavenging activity against stable DPPH using method reported by Blois with slight modifications [22]. Briefly, 20 µL of test sample was mixed with 180 µL of 167 µM DPPH (1,1-Diphenyl-2-Picrylhydrazyl Radical) solution. The reaction was carried out in the dark for 30 min at room temperature. Then the absorbance was measured at 520 nm by using DTX-880 Multimode Detector. *% Inhibition* was calculated using the following equation; *% Inhibition* = $\{[(PC - NC) - (S - B)]/(PC - NC)\} \times 100$, when PC is an absorbance of 20 µL of acetone and 180 µL of 167 µM DPPH mixture, NC is an absorbance of 200 µL of acetone, S is an absorbance of 20 µL of test sample and 180 µL of 167 µM DPPH mixture, and B is an absorbance of 20 µL of test sample and 180 µL of acetone mixture. The experiment was done in triplicate.

Inhibition of lipid peroxidation by the ferric thiocyanate

Pomelo oils and microemulsion were test for the inhibition of lipid peroxidation by the ferric thiocyanate using method reported by Niehius with slight modifications [23]. Briefly, 100 µL of test sample was mixed with 1 mL of 25 mM linoeic acid in acetone and 1 mL of 0.1 M phosphate buffer pH 7.0 in the test tube with cork lid stock. The reaction was carried out in the dark for 6 h at 60°C. Then 50 µL of the mixture was mixed with 3 mL of 75% EtOH, 20 µL of 35% ammonium thiocyanate, and 20 µL of 20 mM ferrous chloride in 3.5% HCl. After mixing by vortex mixture until homogeneous for 1 min, the absorbance was measured at 500 nm by using UV-Visible spectrophotometer. *% Inhibition* was calculated using the following equation; *% Inhibition* = $[(B - S)/B)] \times 100$, when B is an absorbance of the mixture of 100 µL of acetone, 1 mL of 25 mM linoeic acid in acetone, and 1 mL of 0.1 M phosphate buffer pH 7.0 in the absence of test sample and S is an absorbance of 1 mL of 25 mM linoeic acid in acetone, and 1 mL of 0.1 M phosphate buffer pH 7.0 in the presence of 100 µL of test sample. The experiment was done in triplicate.

Mushroom tyrosinase inhibitory assay

Pomelo oils and microemulsion were test for the inhibition against tyrosinase using the method from Pomerantz with slight modifications [24]. Briefly, 100 µL of each samples was mixed with 40 µL of 2.5 mM L-tyrosine solution. After 5 min of incubation at 37°C, 60 µL of 50 units/mL of mushroom tyrosinase was added. The generated dopachrome was then determined after 15 min of incubation at 37°C by the absorbance measurement at 450 nm using multimode detector. The percentage inhibition was calculated using the following equation; *% inhibition* = $[1 - (S / C)] \times 100$, when S is an absorbance of the solution with sample and C is an absorbance of the solution without sample. The experiment was done in triplicate.

Cytotoxicity

The effect of pomelo oils on cell viability of peripheral blood mononuclear cell (PBMC) was determined by using a colorimetric technique, which was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [25].

PBMC isolation

Blood (20-25 mL) was taken from same donor throughout the research by using the 25 mL syringe. The blood sample was diluted with the same volume of phosphate buffer saline (PBS). After that, the diluted blood sample was carefully layered on Ficoll-Paque Plus. Then the mixture was centrifuged under at 5000 × g for 30 min at 18-20°C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pelleted down with three volumes of PBS for twice and resuspended RPMI-1640 media with 100 IU/mL of penicillin, 100 µg/mL of streptomycin, 10% v/v fetal bovine serum (FBS). Cell counting was performed to determine the PBMC cell number with equal volume of trypan blue.

Cell viability assay

The effect of the pomelo oils on cell viability of PBMC was determined by using a colorimetric technique (MTT assay). Briefly, 100 µL of PBMC with cell concentration at 10^5cells/mL was added into all wells in the 96-well plate and incubate in 37°C, 5% CO_2 and 90% humidity incubator for 24 hr. Then 100 µL of various concentrations of the extract was added to the cells compared with untreated cells and incubated again in the same condition for 48 hr.

After the corresponding period, 100 µL of media was removed from each well and 25 µL of MTT at 5 mg/mL was added into each well and incubated again for 4 hr. All the media was removed by turning the 96-well plate upside down. Then 200 µL of dimethyl sulfoxide (DMSO) was added to each well to extract and solubilize the formazan crystal. Finally, the plate was read at 540 nm by using microplate reader. All the experiment was done at least twice.

Microemulsion formulation

Pseudoternary phase diagram construction

Pseudoternary phase diagrams of pomelo oils were constructed using a slightly modified water titration method [26]. Various non-ionic surfactants (Tween 20, Tween 80, Triton X-100, Triton X-114, or Span 80) were mixed with a co-surfactant (ethanol, propan-2-ol, glycerine, PG, or PEG-400) at a weight ratio of 1:2, 1:1, 2:1, or 4:1 to obtain surfactant mixture (Smix).

The essential oils and Smix were then mixed at various weight ratios (0:1, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 1:0) and the resulting mixtures were subsequently titrated with water under moderate agitation at room temperature. The samples were classified as microemulsion when they appeared visually as clear liquids.

The different formulations were made in triplicate. The pseudoternary phase diagrams were drawn by Origin Pro 8 program. The ME regions were measured by ImageJ 1.47v program.

Characterization of microemulsion

Photon correlation spectroscopy

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer® version 5.00, Malvern Instruments Ltd, Malvern, UK).

The sizing measurements were carried out at a fixed angle of 173°. The reported results are the mean and standard deviation (S. D.) of at least ten measurements on the sample.

Rheology study

Viscosity of the microemulsions was measured using a Brookfield DVIII rheometer (Brookfield Engineering Laboratories, Stroughton, MA) fitted with a bob spindle. Brookfield Rheocalc operating software was used to control the measurement. A sample volume of 70 mL was used. The measurements were performed in triplicate at 25°C.

Statistical analysis

All data were demonstrated as a mean±standard deviation (S. D.). Individual differences were evaluated by One-Way ANOVA: post-hoc test. In all cases, $p<0.05$ indicated significance.

RESULTS AND DISCUSSION

Yield and density

Essential oils from peel of pomelo are light yellow liquid with their individual characteristic odor. Yield and density of the oils are shown in **Table 1**. KN shows the highest yield among 4 subspecies in this study. The densities of all oils were not different.

Table 1: Yield and density of essential oil from various subspecies of pomelo

Subspecies	%Yield (mL/g)	Density (g/mL)
KN	0.188	0.86
KP	0.172	0.86
KT	0.177	0.86
KY	0.185	0.86

Table 2: Chemical composition for the essential oils from various subspecies of pomelo

No.	RT	Compound	% Area				KI	
			KN	KP	KT	KY	Sample	Ref[a]
1	4.13	alpha-pipene	0.80	0.44	0.76	0.54	939	939
2	5.00	sabinene	0.39	0.27	0.52	0.29	977	975
3	5.10	beta-pinene	0.59	0.89	2.07	1.16	981	979
4	5.40	beta-myrcene	2.93	2.63	2.59	2.55	993	991
5	5.67	alpha-phellandrene	0.11	0.14	0.03	0.03	1003	1003
6	6.66	limonene	86.19	85.76	79.36	80.20	1035	1029
7	7.15	trans-beta-ocimene	0.43	0.56	0.47	0.52	1050	1050
8	7.49	gamma-terpinene	0.05	0.06	0.10	0.07	1058	1060
9	7.98	trans-linalool oxide	0.38	0.36	0.59	0.30	1072	1073
10	8.47	cis-linalool oxide	0.01	0.14	0.02	0.02	1083	1087
11	9.02	alpha-terpinolene	0.93	1.39	1.69	1.18	1098	1089
12	10.34	trans-p-mentha-2,8-dien-1-ol	0.23	0.24	0.33	0.29	1124	1123
13	10.51	ocimene	0.06	0.04	0.04	0.02	1133	1132
14	11.92	terpinen-4-ol	0.14	0.18	0.41	0.20	1178	1177
15	12.56	alpha-terpineol	0.88	-	1.92	0.72	1196	1189
16	13.75	trans-carveol	0.10	0.16	0.19	0.11	1202	1217
17	14.10	cis-carveol	0.22	0.09	0.32	0.11	1231	1229
18	14.25	nerol	-	0.20	0.40	0.20	1232	1230
19	14.52	neral	0.52	0.63	1.18	0.36	1237	1238
20	15.21	geraniol	0.30	0.10	0.41	0.12	1254	1253
21	15.77	geranial	-	0.87	1.78	0.52	1270	1267
22	18.24	hexyl triglate	0.03	-	0.11	-	1337	1333
23	19.87	alpha-copaene	-	-	-	0.08	1376	1377
24	20.44	geranyl acetate	0.09	0.21	0.08	0.14	1388	1381
25	20.61	beta-elemene	0.09	0.06	0.16	0.04	1392	1391
26	21.43	methyl eugenol	0.04	0.03	0.08	0.09	1410	1404
27	21.52	beta-caryophyllene	-	-	-	0.11	1415	1409
28	21.63	trans-caryophyllene	0.14	0.14	0.11	-	1418	1419
29	24.13	germacrene d	-	-	-	0.10	1481	1485
30	24.62	valencene	0.34	0.14	0.60	0.33	1492	1496
31	24.73	bicyclogermacrene	0.11	-	-	-	1494	1500
32	25.36	(E,E)-alpha-farnesene	-	0.17	0.10	0.17	1509	1506
33	25.56	7-epi-alpha-selinene	0.11	0.07	0.14	0.12	1516	1522
34	27.60	nerolidol	-	0.11	0.19	0.09	1564	1563
35	33.44	(E,E)-farnesol	0.10	0.26	0.66	0.12	1728	1725
36	36.24	nootkatone	1.92	-	2.54	1.34	1807	1807
		Total	**98.26**	**96.33**	**99.95**	**92.26**		

RT=Retention time, KI=Kovat's index,a=[21]

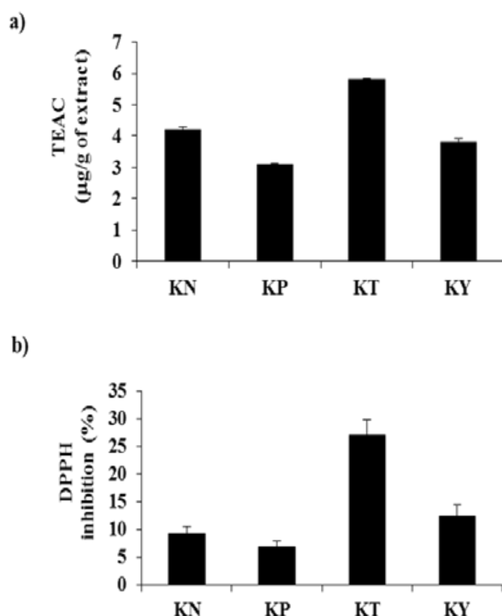
GC-MS

Relative amounts of the individual compounds of each oils were presented as peak area percentage of the total peak area as shown in **Table 2.** The GC-MS data indicated that 29, 29, 32, and 33 compounds, making up 98.26%, 96.33%, 99.95%, and 92.26% of the total oil composition were from KN, KP, KT, and KY oil, respectively. The GC-MS data obviously indicated that limonene was the most abundant volatile composition in this plant with the percentage of 86.19, 85.76, 79.36, and 80.2 in KN, KP, KT, and KY oil, respectively. The results were in a good acceptance with the previous study reported that limonene showed the greatest amount in volatile oil of pomelo peel [27, 28].

Antioxidant activity

Previous studies demonstrated that antioxidant activity was depended on the method used and recommended to base the conclusions on at least two different test methods [29-31]. Antioxidant activities of essential oil from various subspecies of pomelo were evaluated by means of trolox equivalent antioxidant capacity (TEAC), DPPH inhibition, and lipid peroxidation inhibition and the results are shown in Figure 1. The test systems using a stable free radical including DPPH and ABTS give information on the radical scavenging or antiradical activity [32]. The results from ABTS assay were reported as TEAC values. Among 4 subspecies of pomelo, KT oil shows the highest TEAC value indicating the highest free radical scavenging activity. The results were in a very good agreement with the DPPH assay indicating that KT oil shows the highest radical scavenging activity. The test systems using a lipid peroxidation, which is the most studied biologically relevant free radical chain reaction, give information on the antioxidant activity [33]. Lipids are oxidized by several mechanisms including free radical-mediated oxidation and the antioxidants are able to inhibit the lipid peroxidation and the deleterious effects caused by the lipid peroxidation products [34]. Among 4 subspecies of pomelo, KT oil also shows the highest inhibition against lipid peroxidation indicating the highest antioxidant activity.

Since the essential oils are complex combination of several terpenes, the possible synergism might occur, especially in the case of KT oil. According to the compositions of each oils, some terpenes were found more abundant in KT oil than the others such as beta-pinene (2.07%), alpha-terpineol (1.92%), geranial (1.78%), and terpinen-4-ol (0.41%). However, the previous study shows no antioxidant activity of the isolated terpenes including beta-pinene [35]. Therefore, the synergism of these terpenes would be a key reason responsibles for their radical scavenging and antioxidant activities.



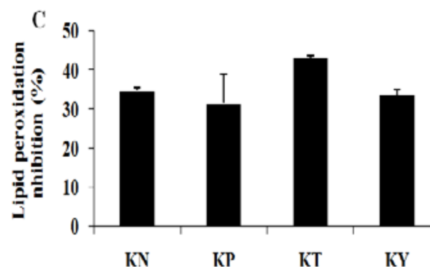


Fig. 1: TEAC value (a), DPPH inhibition (b), and lipid peroxidation inhibition (C) of essential oil from Kao-Namphung (KN), Kao-Puang (KP), Kao-Tang-Gwa (KT), and Kao-Yai (KY).

Antityrosinase activity

The antityrosinase activities are not statistically different among KT oil, KN oil, and KP oil, whereas, KY oil shows a significant lower inhibition ($p<0.05$) as shown in Figure 2. Recently, there were studies reported antityrosinase activity of methanolic extract of pummelo peel and fresh pomelo juice [36,37]. But in the present study, antityrosinase activity of the pomelo oils were very low compared to the commercially used antioxidants, L-ascorbic acid ($IC_{50} = 88.44 \pm 0.62$ μg/mL).

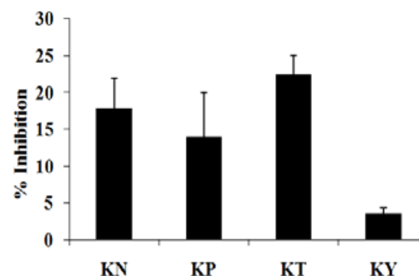


Fig. 2: Inhibitory activity of 5% essential oil from Kao-Namphung (KN), Kao-Puang (KP), Kao-Tang-Gwa (KT), and Kao-Yai (KY) on converting L-tyrosine to L-dopa by tyrosinase.

Cytotoxicity

As KT oil exhibited the highest antioxidant and antityrosinase activity, it was selected for the further in depth study. The cell viability of human PBMCs after exposure to KT oil for 48 h is shown in Figure 3. It is noted that KT oil was very safe since it had no toxic effect on human PBMCs for a nearly 100% of cell viability were observed even at high concentration (50 %v/v) were used.

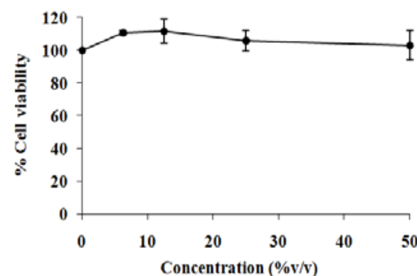


Fig. 3: Dose-response curve of viability of PBMC versus concentration of KToil.

Pseudoternary phase diagram construction

Nonionic surfactants were used in microemulsion formulation in this study because of their safety and less irritation [38]. Pseudoternary phase diagrams showing the effect of surfactant type are shown in Figure 4. Tween 80 and Span 80 gave no

microemulsion region in the phase diagram, whereas, Tween 20, Triton X-114, and Triton X-100 gave microemulsion regions of 33.8%, 23.1%, and 15.6%, respectively. Liquid crystal systems observed as viscous gel were found in the system of Triton X-114 and Triton X-100 [39,40]. Since Tween 20 gave the largest area of microemulsionregion, it was selected for the further study.

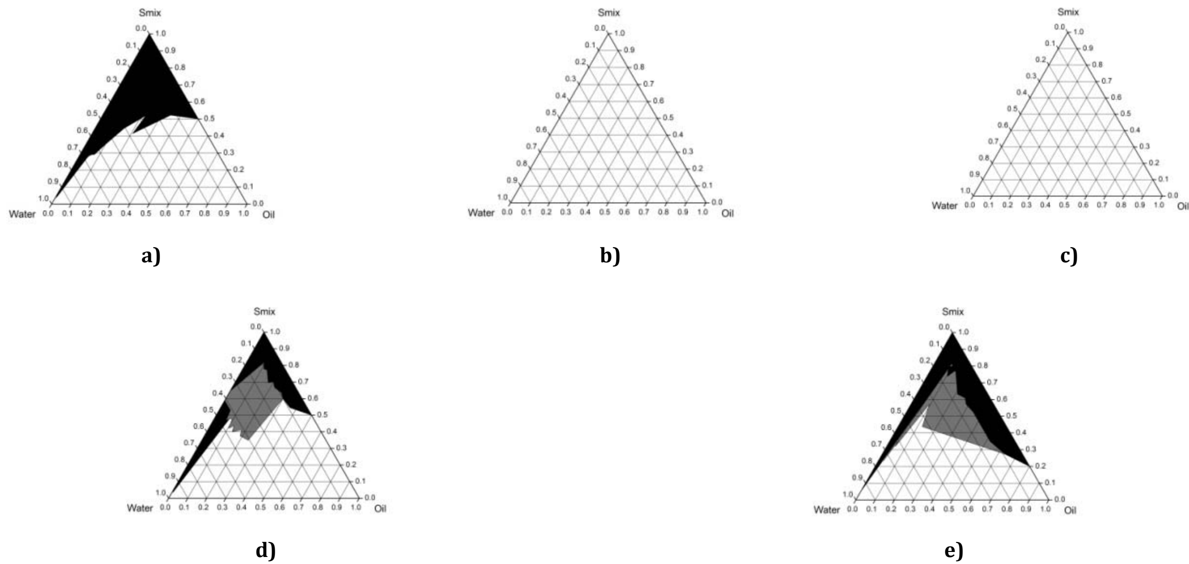


Fig. 4: Pseudoternary phase diagram showing microemulsion region (balck) and liquid crystal region (gray) of KT oil/surfactant/PG/water when the surfactant were Tween 20 (a), Tween 80 (b), Span 80(c), Triton X-100 (d), and Triton X-114 (e).

The effects of co-surfactant on microemulsion formation are shown in Figure 5. Microemulsion regions of the system using PEG 400, PG, ethanol, propa-2-ol, and glycerin were 22.8%, 19.8%, 16.2%, 19.2%, and 19.4%, respectively. The results were in a good agreement with the previous study reporting that co-surfactant type showed an obvious effect on the microemulsion formation [41,42]. Since PEG 400 gave the largest microemulsion region in the phase diagram, it was selected as a co-surfactant in the further system.

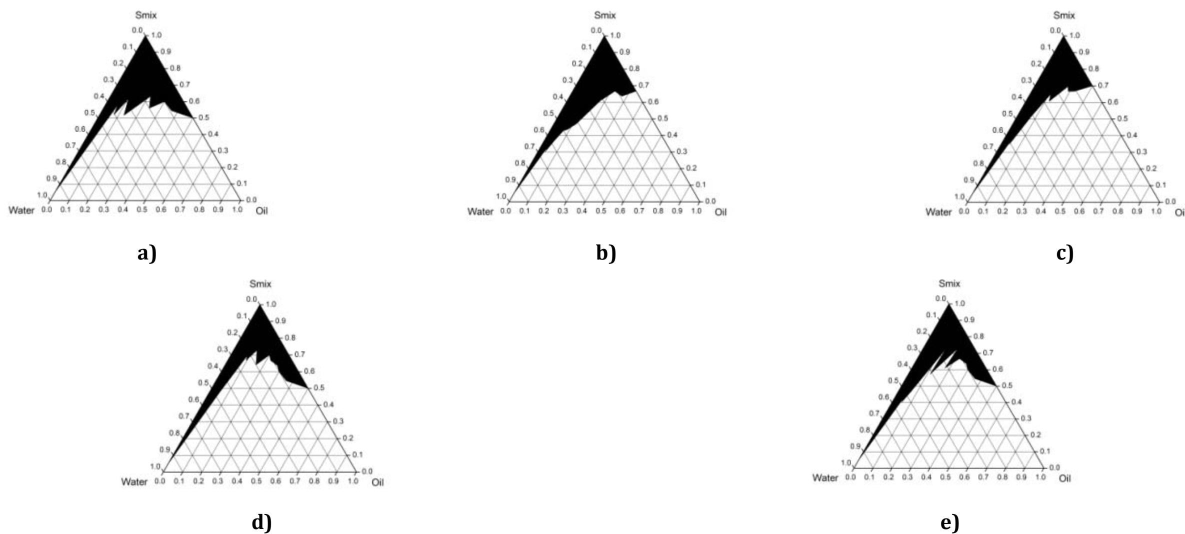


Fig. 5: Pseudoternary phase diagram showing microemulsion region (balck) of KT oil/Tween 20/co-surfactant/water when the co-surfactants were PEG 400 (a), PG (b), ethanol (c), propan-2-ol (d), and glycerin(e).

The effects of surfactant to co-surfactant ratio are shown in Figure 6 when Tween 20 and PEG 400 were used as surfactant and co-surfactant, respectively. It is noted that higher proportion of surfactant gave higher microemulsion region. The surfactant to co-surfactant ratio of 4:1, 2:1, 1:1, and 1 :2 gave the region of 36.3%, 22.8%, 20.9%, and15.2%, respectively. The results were in a good acceptance with the previous study reported that higher amount of polyoxyethylated castor oil was able to incorporated in microemulsion when the ratio of surfactant to co-surfactant (Cremophor EL®:Transcutol®) increased [43]. Kale and Allen also reported that the increasing of surfactant to co-surfactant ratio could increase the microemulsion formation of mineral oil using Brij 96 as a surfactant, and glycerin, ethylene glycol, and propylene glycolas co-surfactants[44]. Since the ratio of 4:1 gave the largest region of microemulsion, it was selected for the further studies.

Characterizationof microemulsion

According to the above mentioned results, the system of KT oil/Tween 20/PEG 400/water was selected for the further studies (Figure 7). ME represents to the formulation containing 15% KT oil, 36% Tween 20, 9% PEG 400, and 40% water. The particle size of ME analyzed by photon correlation spectroscopy was 90.28 ± 1.60 nm which is in a range of microemulsion leading the formulationto be transparent. Furthermore, ME showed the Newtonian flow behavior with theviscosity of 16.78 ± 0.12 Pas confirming the formation of microemulsion [45-47].

The radical scavenging and antioxidant activities of ME were investigated in a comparison with KT oil by DPPH and lipid peroxidation assay, respectively. The results as shown in Figure 8 indicated that ME could enhance both scavenging and antioxidant activities of KT oil. The DPPH inhibition was increased by 42.63%, whereas, the linoleic peroxidation inhibition was increased by 61.15%. These were in a good agreement with our previous studies reported the enhancement of anticholinesterase activity of essential oils from *Cymbopogon citrates* and *Zingiber cassumunar* by using a microemulsion formulation [26,48].

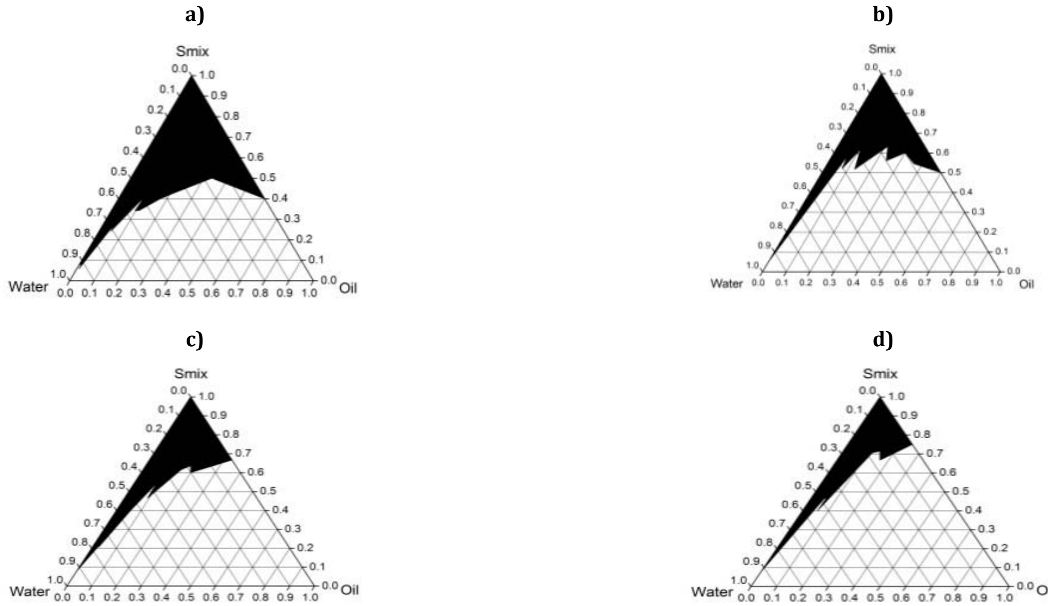


Fig. 6: Pseudoternary phase diagram showing microemulsion region (balck) of KToil/Tween 20/PEG 400/water when the surfactant to co-surfactant ratio were 4:1 (a), 2:1 (b), 1:1 (c), and 1:2 (d).

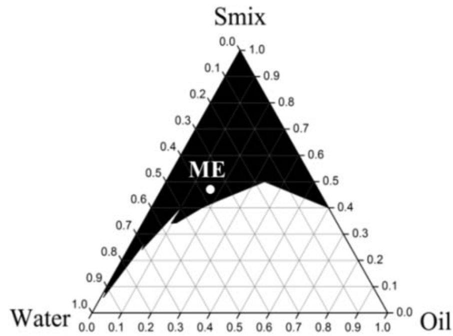


Fig. 7: Pseudoternary phase diagram showing microemulsion region (balck) of KT oil/Tween 20/PEG 400/water when the surfactant to co-surfactant was 4:1. ME is the microemulsion containing 15% KT oil, 36% Tween 20, 9% PEG 400, and 40% water.

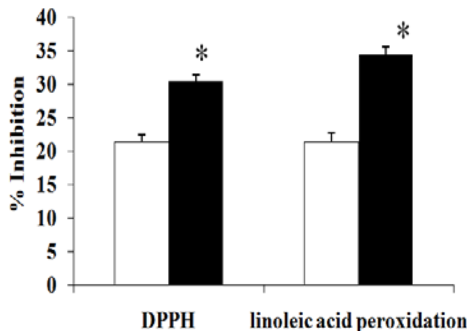


Fig. 8: Inhibitory activities against DPPH and lipid peroxidation of 15% KT oil (□) and ME (■) (* denotes *p*< 0.01).

CONCLUSION

The essential oils from four subspecies of pomelo including KN, KP, KT, and KY were extracted by hydrodistillation. The physical appearance of each oils was not different except their individual specific odor. Their major constituents analyzed by GC-MS was limonene, which was found up to 86.19%, 85.76%, 79.36%, and 80.20% in KN oil, KP oil, KT oil, and KY oil, respectively. Among these oils, KT oil showed the highest radical scavenging and antioxidant activities and also revealed the highest antityrosinase activity on converting L-tyrosine to L-dopa. Moreover, KT oil was friendly to human cells since it showed no *in vitro* toxic effect on human PBMCs. Therefore, microemulsions of KT oil were formulated. The pseudoternary phase diagrams were constructed to provide the information of suitable microemulsion formulations. The effect of surfactant, co-surfactant, surfactant to co-surfactant ratio were also investigated. ME, the microemulsion containing 15% KT oil, 36% Tween 20, 9% PEG 400, and 40% water, were formulated and chracterized. It was a transparent liquid exhibiting the Newtonian flow behavior with low viscosity (16.78 ± 0.12 Pas). In a comparison with KT oil, ME show significantly higher radical scavenging and antioxidant activities (*p*< 0.01). Therefore, ME is an attractive system for further *in vivo* tests and effective topical products development.

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