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Characterization of potent anticholinesterase plant oil based microemulsion

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ABSTRACT

In the present study, essential oils of three edible Thai plants, *Cymbopogon citratus* (Gramineae), *Citrus hystrix* (Rutaceae) and *Zingiber cassumunar* (Zingiberaceae) were comparatively tested for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities using Ellman's colorimetric method. *C. citratus* oil exhibited the highest activity with IC_{50} values of 0.34 ± 0.07 μl/ml and 2.14 ± 0.18 μl/ml against BChE and AChE activity, respectively. It was further investigated whether microemulsions of this oil could be obtained. The effects of type of surfactant and co-surfactant as well as pH and ionic strength on the phase behavior of the oil/water system were investigated. Brij 97, Triton X-114, Tween 20 and Tween 85 were employed as surfactant whereas ethanol and hexanol were used as cosurfactants. The size analysis, electrical conductivity measurements and cholinesterase inhibition assays were done in selected microemulsion. The results revealed that the type and concentration of surfactant and co-surfactant exhibited a distinct influence on the *C. citratus* oil microemulsions. Moreover, the inhibitory activities of the microemulsion formulation were remarkable.

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1. Introduction

Alzheimer's disease (AD), a neurodegenerative disorder, affects an estimated number of more than 18 million elderly people worldwide. This disease is associated with intellectual misfunction and subsequent decline in cognitive, behavioral and motor functions (Farfara et al., 2008). Increased levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) have been found in postmortem brain samples of AD patients which has lead to the hypothesis that the cognitive decline in AD patients is related to progressive cholinergic degeneration (Snyder et al., 2001). Therefore, promising approaches for treating AD are to enhance acetylcholine concentrations in the brain using cholinesterase inhibitors (Scarpini et al., 2003). During the past decade, synthetic inhibitors of AChE and BChE have been clinically evaluated (Mercier et al., 2007; Lefevre et al., 2008). Nevertheless, none of them can cease the disease. Consequently, there is still a great demand for new drug candidates for AD treatment. Particularly, natural sources might be used to isolate such compounds. Many essential oils from medicinal plants show a great variety of biological activities such as antioxidant, antimicrobial, anti-inflammatory and anticancer (Ordentlich et al., 1993; Chatterjee et al., 2004; Rauber et al., 2005). Transdermal delivery has shown to give a sustained drug concentration in the circulation with lower fluctuations as compared to conventional oral drug delivery. Therefore, transdermal delivery is an attractive option for administration of AD drugs (Amzal and Appel-Dingemanse, 2007; Mercier et al., 2007). Terpenoid compounds derived from various essential oils were reported to have low skin irritancy and low systemic toxicity as well as good penetration enhancing activity for both hydrophilic and lipophilic drugs (Pudil et al., 1998; Pan et al., 2008; Kovarik et al., 1999). Therefore, essential oils from edible plants are attractive sources for active agents to treat AD patients via the transdermal route. Microemulsions are isotropic colloidal systems that are formed spontaneously from appropriate combinations of oil, water and surfactant/co-surfactant mixtures (Perry et al., 1978; Greig et al., 2004). They are optically transparent since their internal phase droplet size ranges from 5 to 100 nm (Pedro et al., 2009; Sinico et al., 2005), which is below the wavelength of visible light. They have become of interest for pharmaceutical applications as carrier systems in transdermal drug delivery (Leimann et al., 2009; Holmberg et al., 1998; Moulik and Paul, 1998; García et al., 2001) as they provide several advantages over conventional topical formulations such as creams, ointments and gels (Lawrence and Rees, 2000; Paolino et al., 2002). The manufacturing of microemulsions is easy and the products are thermodynamically stable and therefore have a good pharmaceutical shelf-life. Their flexibility in composition enables microemulsions to solubilize both hydrophobic and hydrophilic compounds, depending on the type of microemulsion used. Moreover, the skin permeation rate of active compounds from microemulsions can be well controlled by the type and ratio of the components (Ingkaninan et al., 2006; Blanco et al., 2009).

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The purposes of this study were to investigate whether essential oils isolated from edible plants possess high anticholinesterase activity and to identify a suitable microemulsion system containing the active oil for topical applications. The phase behavior of different microemulsions was explored as a function of the formulation parameters, among which the type and concentration of surfactant, the oil/water ratio, and the pH and ionic strength of the aqueous phase. A suitable system of each surfactant was selected for size analysis. Moreover, conductivity measurements were done to establish whether o/w or w/o (micro)emulsions were formed.

2. Experimental

2.1. Materials

2.1.1. Plant materials

Three aromatic Thai edible plants, *Cymbopogon citratus* (Gramineae), *Citrus hystrix* (Rutaceae) and *Zingiber cassumunar* (Zingiberaceae) were collected from Chiang Mai, Thailand during January 2009. All three plants were authenticated and voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand.

2.1.2. Chemicals and enzymes

Acetylcholinesterase (AChE, specific activity 425.94 U/mg) from *electrophorus electricus*, butyrylcholinesterase (BChE, specific activity 7.4 U/mg) from equine serum, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), polyoxyethylene 10 oleyl ether (Brij 97), polyethylene glycol sorbitan trioleate (Tween 85), and polyoxyethylene sorbitan monolaurate (Tween 20) were from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were from Fluka (Steinheim, Garmany). Octylphenoxy polyethoxy ethanol (Triton X-114) was from Acros Organics (NJ, USA). Methanol, ethanol and hexanol were analytical grade from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Extraction of the essential oils

The fresh overground part of *C. citratus*, leaves of *C. hystrix* and rhizomes of *Z. cassumunar* were separately cut into small pieces and subjected to hydrodistillation for 3 h using a clevenger type apparatus for oil extraction. The extracted essential oils were collected and stored in a refrigerator and protected from light until further use.

2.2.2. Cholinesterase activity determination

Two types of cholinesterase enzymes, electric eel AChE and horse serum BChE, were used whereas ATCI and BTCI were used as substrates, respectively. The enzyme inhibitory action was done by means of Ellman's method. Briefly, 50 μl of 50 mM Tris–HCl buffer pH 8.0, 25 μl of 1.5 mM ATCI or BTCI, 125 μl of 3 mM DTNB and 2.5 μl of the extracted oils in Tris–HCl buffer containing 10% methanol were mixed orderly. Then, 25 μl of 0.25 U/ml AChE or 0.91 U/ml BChE was added and the reaction was spectrophotometrically followed for 2 min at 415 nm by a microplate reader (Bio-Rad Laboratories Ltd., Japan). In the case of inhibitory activities evaluation of microemulsion, 25 μl of microemulsion containing 10% of the extracted oil was added instead of the extracted oil in Tris–HCl buffer with 10% methanol. The experiments were done in triplicate. The slope of the plot of absorbance versus time was taken as the enzymatic reaction rate. The enzyme inhibitory activity (Inh) was calculated as $1-(Vs/Vb)$, in which Vs is the mean reaction rate in the presence of a certain concentration of the oils and Vb is the mean reaction rate in absence of the oils. IC_{50} values were statistically evaluated using the Graphpad/Prism program.

2.2.3. Construction of phase diagrams

Pseudoternary phase diagrams of the essential oils were constructed using a water titration method. Four surfactants (Brij 97, Triton X-114, Tween 20, and Tween 85) were mixed with a cosurfactant (ethanol or hexanol) at a weight ratio of 1:2, 1:1, or 2:1 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. Instead of water, citrate buffers of pH 4.0 (composed of 0.1 M citric acid and 0.1 M sodium citrate), phosphate buffers of pH 6.0 and 8.0 (composed of 0.2 M monosodium phosphate and 0.2 M disodium hydrogen phosphate) and solutions of 0.1, 0.5 and 1.0 M sodium chloride and calcium chloride were also used to investigate the effect of pH and ionic strength on the phase diagrams, respectively. The different formulations were made in triplicate. The pseudoternary phase diagrams were drawn by SigmaPlot for Windows version 10.0.

2.2.4. Electric conductivity measurements

The electrical conductivity of microemulsions comprising *C. citratus* oil and Tween 20/ethanol (2:1) mixtures in different ratio (10:90, 20:80, 30:70 and 40:60) was measured using 100 mM NaCl solution as aqueous phase. The conductivity was measured by Cyberscan CON 11: hand-held conductivity meter (Eutech Instruments, Singapore) using conductivity/TDS electrode cell. The experiment was performed at $25 \pm 1.0\,^{\circ}C$ by dipping the electrode into the test sample until equilibrium was reached and reading became stable. The measurements were done in triplicate.

2.2.5. 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 90°. The reported results are the mean and standard deviation (S.D.) of at least ten measurements on the sample.

2.2.6. Statistical analysis

All data were demonstrated as a mean ± S.D. The inhibitory activity of the oil samples on AChE and BChE is presented as % inhibition and IC_{50} values. Individual differences were evaluated by One-Way ANOVA: post hoc test. In all cases, $p < 0.05$ indicates significance.

3. Results and discussion

3.1. Inhibition of cholinesterase activity by essential oils

Three tropical plants include *C. citratus*, *C. hystrix* and *Z. cassumunar* were selected for studying the bioactivities of their essential oils, which are all used both in foods and cosmetics (Han et al., 2005; Craig et al., 2006; Akhila, 2009). *C. citratus* which has been used in fresh, dried and powdered forms is widely cultivated in the tropical regions. The fresh stalks are normally found on Asian markets as well in many health food markets. This aromatic herb is used in many types of Caribbean and Asian cooking and its essential oil is commercially used in soaps, perfumes, cosmetics and in aromatherapy as a relaxant (Akhila, 2009). In South East Asia, *C. citratus* has been used in Ayurvedic herbalism to combat depression, fever, digestive disorders and pain management (Akhila, 2009; Steflitsch and Steflitsch, 2008). Another plant, *C. hystrix*, is widely

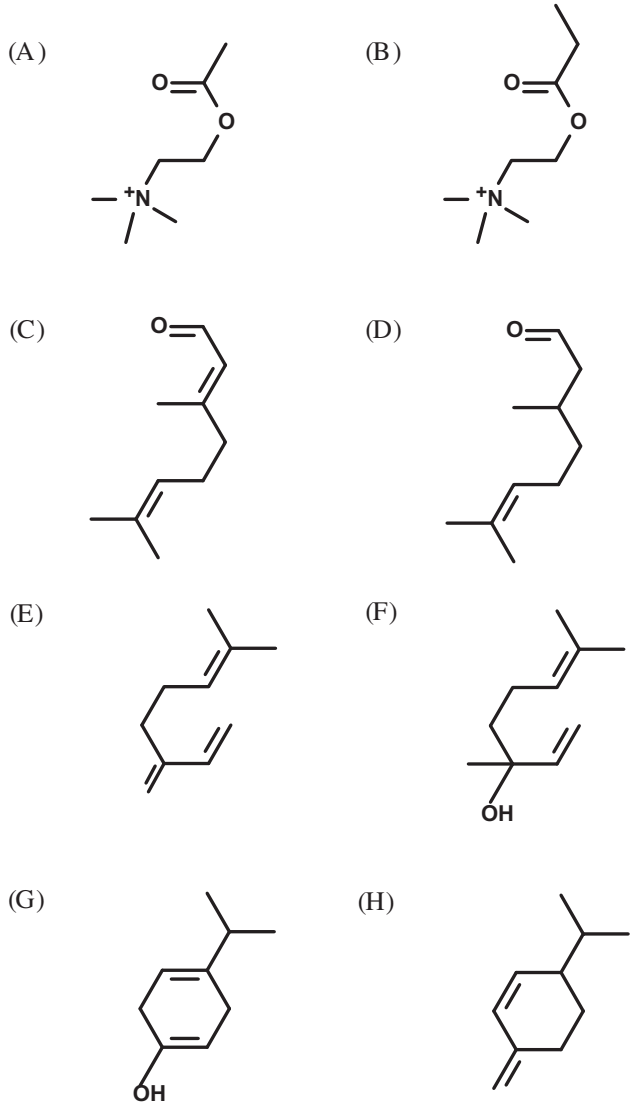

Fig. 1. Chemical structures of acetylcholine (A), butyrylcholine (B), citral (C), citronellal (D), beta-myrcene (E), linalool (F), terpinene-4-ol (G) and beta-phellandrene (H).

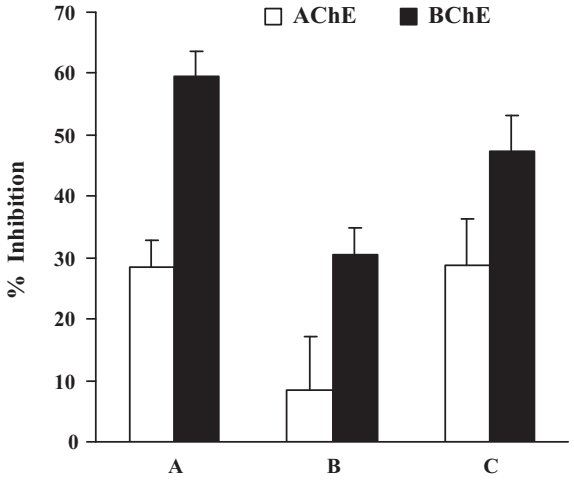

Fig. 2. Inhibitory activities of *C. citratus* oil (A), *C. hystrix* oil (B), and *Z. cassumunar* oil (C), 2.5 μl, against AChE and BChE.

distributed and cultivated worldwide. The fruits and leaves are normally used as natural flavor in various foods and beverages in Asian countries (Hanny and George, 1995) and their aromatic oils are used in aromatherapy and cosmetic industries (Craig et al., 2006). *Z. cassumunar* is a tropical ginger that has been widely distributed in Southeast Asia. Its rhizomes have been traditionally used for gastrointestinal distress and to motion sickness prevention (Han et al., 2005).

The yields of the essential oils extracted from *C. citratus*, *C. hystrix* and *Z. cassumunar* were 0.24, 0.68, and 0.48% (v/w) of the fresh plant samples respectively. It has been reported that essential oils consist of terpenoids, however, the main compound in the investigated oils is different. *C. citratus* oil mainly consists of citral (Blanco et al., 2009), the principal component of *Z. cassumunar* oil is terpinene-4-ol (Pithayanukul et al., 2007), which is a monocyclic monoterpenoid, whereas that of *C. hystrix* oil comprises acyclic and monocyclic monoterpenoids of citronellal and beta-phellandrene (Pudil et al., 1998). The structures of these compounds are shown in Fig. 1.

The different essential oils demonstrated cholinesterase inhibitory action, as shown in Fig. 2. It was found that their inhibitory action on BChE was stronger than on AChE. Both enzymes are serine hydrolases, which share 65% amino acid sequence homology (Pan et al., 2008). The difference in amino acid residues of the active sites of each enzyme dictates the substrate specificity of the enzymes (Kovarik et al., 1999). The three distinct domains in the active sites of ChE are the choline binding site, the acyl-binding pocket and the peripheral anionic site (Radić et al., 1993). The main differences between AChE and BChE are in the acyl-binding pocket. The two rather bulky hydrophobic amino acid residues Phe288 and Phe290 in the acyl-binding pocket of AChE are replaced by less bulky Leu286 and Val288 residues in BChE (Pan et al., 2008). These differences make it possible for the binding of bulkier substrates to the active site of BChE (Ordentlich et al., 1993). The higher inhibitory activity for BChE can be explained by fact that the terpenoids present in the essential oils, especially those containing acyl-binding groups such as citral, are rather bulky (Fig. 1) are therefore able to bind to BChE with a higher affinity than to AChE. Of the oils tested, *C. citratus* oil showed the highest BChE inhibitory activity 59.4 ± 4.1%; *Z. cassumunar* and *C. hystrix* oils showed the inhibitory activity of 47.5 ± 5.6% and 30.6 ± 4.1%, respectively. *C. citratus* oil demonstrated an inhibition of 28.4 ± 4.4% of the AChE activity which was similar to that of *Z. cassumunar* oil but much higher than that of *C. hystrix* oil (around 10%). From these results, we conclude that of the investigated oils, *C. citratus* oil has the highest cholinesterase inhibitory activity. Therefore, it was selected as an active ingredient in microemulsion formulations. The essential oil from *C. citratus* was further studied for its IC_{50} value against AChE and BChE. Fig. 3 shows the dose-dependent inhibition of AChE and BChE by *C. citratus* oil. The calculated IC_{50} values show that *C. citratus* oil can be characterized as a potent BChE inhibitor with IC_{50} of 0.34 ± 0.07 μl/ml and a moderate AChE inhibitor with IC_{50} of 2.14 ± 0.18 μl/ml. The plateau's in Fig. 3a and b begin at 100 μl/ml and 5 μl/ml, respectively. These plateau's signify that the concentration of *C. citratus* oil over 100 μl/ml and 5 μl/ml are not necessary for AChE and BChE inhibition, respectively. Only 100 μl/ml (0.001%) of *C. citratus* oil in the microemulsion formulations causes inhibition the activities of both enzymes. It was previously reported that the 75% (GC analysis) of *C. citratus* oil is composed of citral and a mixture of geranial and neral (Chatterjee et al., 2004; Rauber et al., 2005). On the other hand, citral is absent in the essential oils obtained from the other two plants. Therefore, the observed high cholinesterase inhibitory activity of *C. citratus* oil can likely be ascribed to citral. Indeed, it has been reported that in humans citral is effective in mild Alzheimer's cases (Guginski et al., 2009;

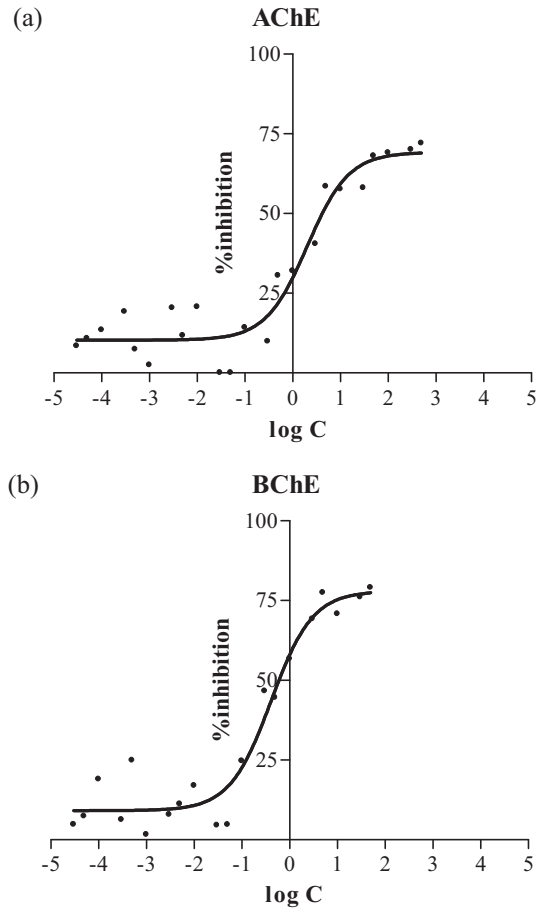

Fig. 3. Dose dependent inhibition of AChE (a) and BChE (b) by *C. citratus* oil.

Adams et al., 2007) and low doses of this compound have shown to improve the learning competency and enhance the memory of rats (Yang et al., 2009). One of the domains in the active site of cholinesterase is the ACh cation-binding pocket and it has been found that hydrophobic compounds can bind herein (Shafferman et al., 2008). Likely, citral can competitively bind in this pocket, preventing that the natural substrate ACh can be bound and enzymatically be cleaved (Radić et al., 1993). *C. citratus* oil also contains several kinds of hydrocarbon skeletal terpenes which also might have affinity to this pocket and consequently contribute to the inhibitory activity of the oils (Mukherjee et al., 2007). Because of the high cholinesterase inhibitory activity, *C. citratus* oil was selected for further development of a microemulsion formulation which recently emerged for transdermal delivery (Sakulku et al., 2009).

3.2. Microemulsion of C. citratus oil: phase diagrams

3.2.1. Influence of surfactant

Four nonionic surfactants (Brij 97, Triton X-114, Tween 20 and Tween 85; structures shown in Fig. 4 were selected for the preparation of microemulsions of *C. citratus* oil because of their low toxicity (Kreilgaard, 2002; Sagitani and Friberg, 1980). Fig. 5 demonstrates the phase diagrams of water/oil/surfactant/co-surfactant systems. Surfactant and co-surfactant (Smix) are apart from oil and water necessary constituents in microemulsions. Increasing amounts of Smix allow the microemulsions to contain greater amount of active oil. However, the types of surfactants also play a dominant role. To prepare microemulsions with the same amount of active oil, different amounts of Smix were needed. For example, 10% of *C. citratus* oil needs about 30%, 45%, 40% and 10% of Smix for Brij 97, Triton X-114, Tween 20 and Tween 85, respectively. Moreover, the area exist in the phase diagram of these four surfactants are obviously distinct from each others. The zone of microemulsions covered 56.3%, 33.0%, 31.5% and 10.0% of the total area of the triangle phase diagrams using Brij 97, Triton X-114, Tween 20 and Tween 85, respectively. Despite the fact that two surfactants having the same HLB (Brij 97 and Triton X-114, Fig. 5a and b) shows differences in pseudoternary phase diagrams. Normally, the lower the CMC of a surfactant the less is needed to form a microemulsion (Ben-Moshe and Magdassi, 2004). Thus at the same concentration, Triton X-114 should be a better surfactant to create microemulsions. Surprisingly, our results show that Brij 97 is a better surfactant to form microemulsions than Triton X-114 although its CMC value is greater, indicating that the molecular geometry of the surfactants, which is very different for these two surfactants, may play a role in microemulsion formation (Baker et al., 1984). However, it is known that more bulky surfactant molecules are able to form films that are more stable against rupture (Babak and Stébé, 2002). This observation can clarify the better ability in microemulsion formation of Brij 97, which has higher molecular weight and is more bulky than Triton X-114. Fig. 5 also shows that the microemulsion forming ability of Tween 85 was much less pronounced than those of the structurally related Tween 20, which may be explained by the higher HLB value of the latter surfactant. Since the HLB of a surfactant has been suggested a parameter to explain the stabilization properties of some microemulsion systems (Li and Kunieda, 2003). However, while it is known that the higher the HLB value of a surfactant, the easier it forms o/w emulsions (Martin, 1961; Drew, 2002), there is no direct correlation between the HLB value of the surfactants and their ability of forming microemulsions. For example, Brij 97 has an HLB value of 12.4 and has the largest microemulsion area in the phase diagrams of this study, whereas the HLB value of Tween 85 is only slightly lower (11.4) but the use of this surfactant resulted in a substantially smaller microemulsion area. Given its good ability to prepare microemulsions with *C. citratus* oil, its low toxicity (Lawrence and Rees, 2000) and its cost effectiveness, Tween 20 was selected for further microemulsion studies.

3.2.2. Influence of co-surfactant and surfactant/co-surfactant ratio

The use of a single surfactants is unlikely to reduce the interfacial tension between oil and water to form stable microemulsions, and therefore the addition of a co-surfactant is generally required (Tenjarla, 1999). It has previously been shown that the type of co-surfactant and the ratio of surfactant/co-surfactant affect the formation of microemulsions (Liu et al., 1998). Ethanol and hexanol were used as co-surfactants in this study. Fig. 6 shows that the microemulsion regions of the systems with ethanol are much larger than those with hexanol. Ethanol is more hydrophilic than hexanol and it is therefore likely that hexanol will be essentially present in the oil phase while ethanol partitions over the aqueous and oil phase. Therefore, the interfacial tension between the oil and aqueous phase in the system with ethanol is lower than that in the corresponding system with hexanol. This in turn favors the formation of small emulsified droplets. Further, it has been reported that ethanol is able to insert into the interfacial layer and forms a tight interfacial film (Yuan et al., 2006). This also might contribute to the better performance of ethanol compared to hexanol to create microemulsions.

3.2.3. Effect of ionic strength and pH

It has been shown that the region of existence of microemulsions as well as the size of the emulsified droplets may be affected by electrolytes (Carlfors et al., 1991). To investigate whether the

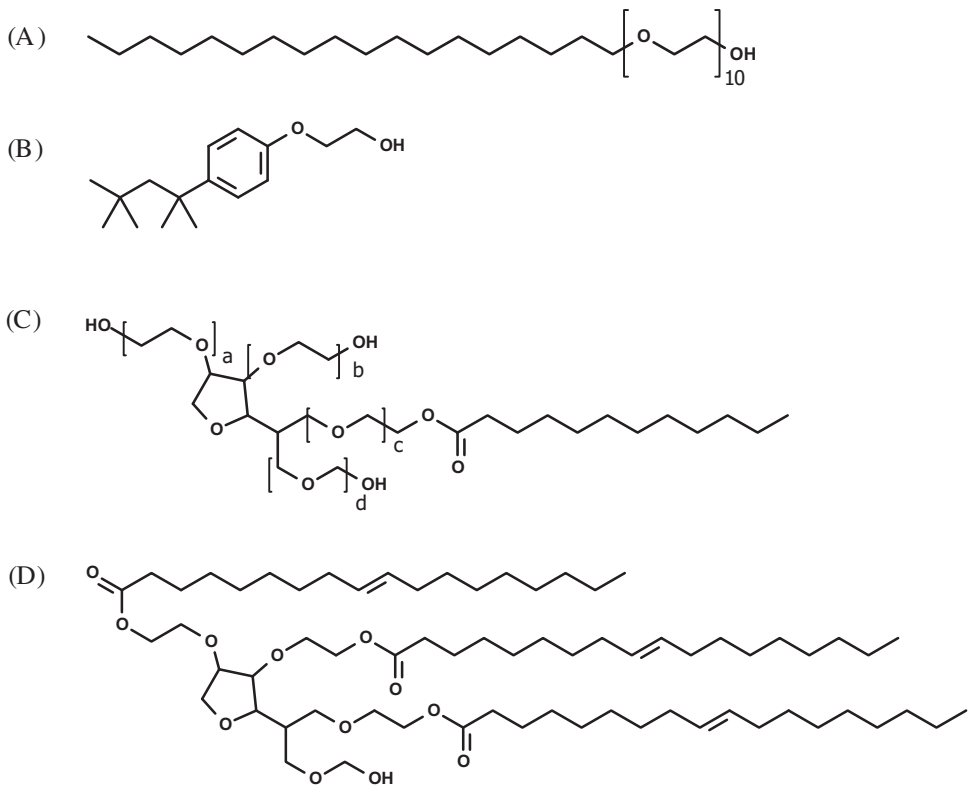

Fig. 4. Chemical structures of the used surfactants: Brij 97 (A), Triton X-114 (B), Tween 20 (C) and Tween 85 (D).

ionic strength of the aqueous phase affected microemulsions of *C. citratus* oil, NaCl and $CaCl_2$ were employed as monovalent and divalent electrolytes, respectively. The results of Fig. 7 show that the phase diagrams of mixtures containing 0.5 M of either NaCl or $CaCl_2$ are not different than that from the mixtures containing no salt. However, at high salt concentration (1.0 M NaCl or $CaCl_2$) the region of existence of microemulsion became smaller. Likely, the PEG chains of Tween 20 are dehydrated in the high salt solution

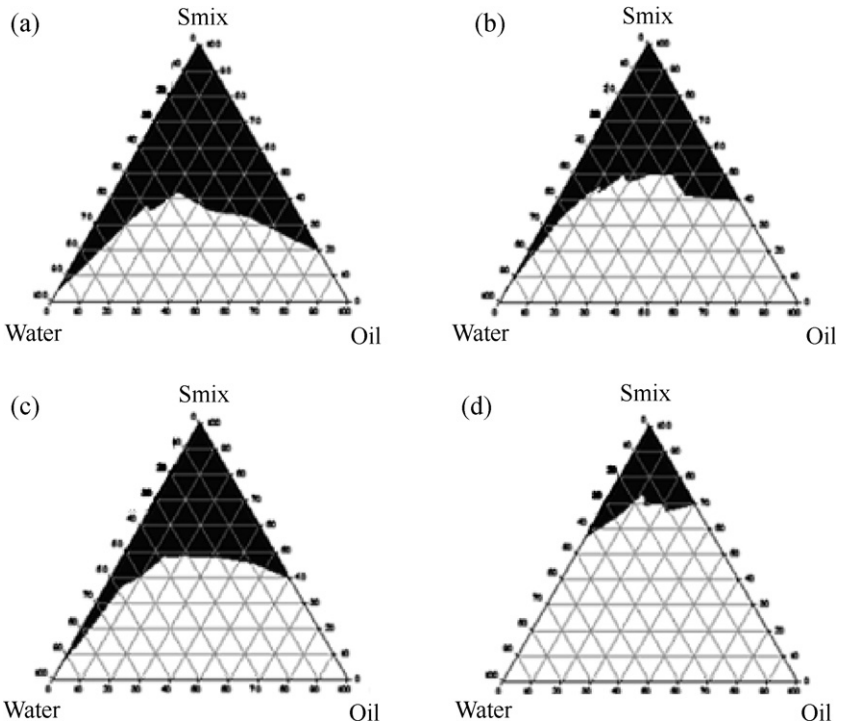

Fig. 5. Pseudoternary phase diagrams of *C. citratus* oil/water/surfactant/co-surfactant mixtures containing Brij 97 (a), Triton X-114 (b), Tween 20 (c) and Tween 85 (d). The dark area represents the region of microemulsion.

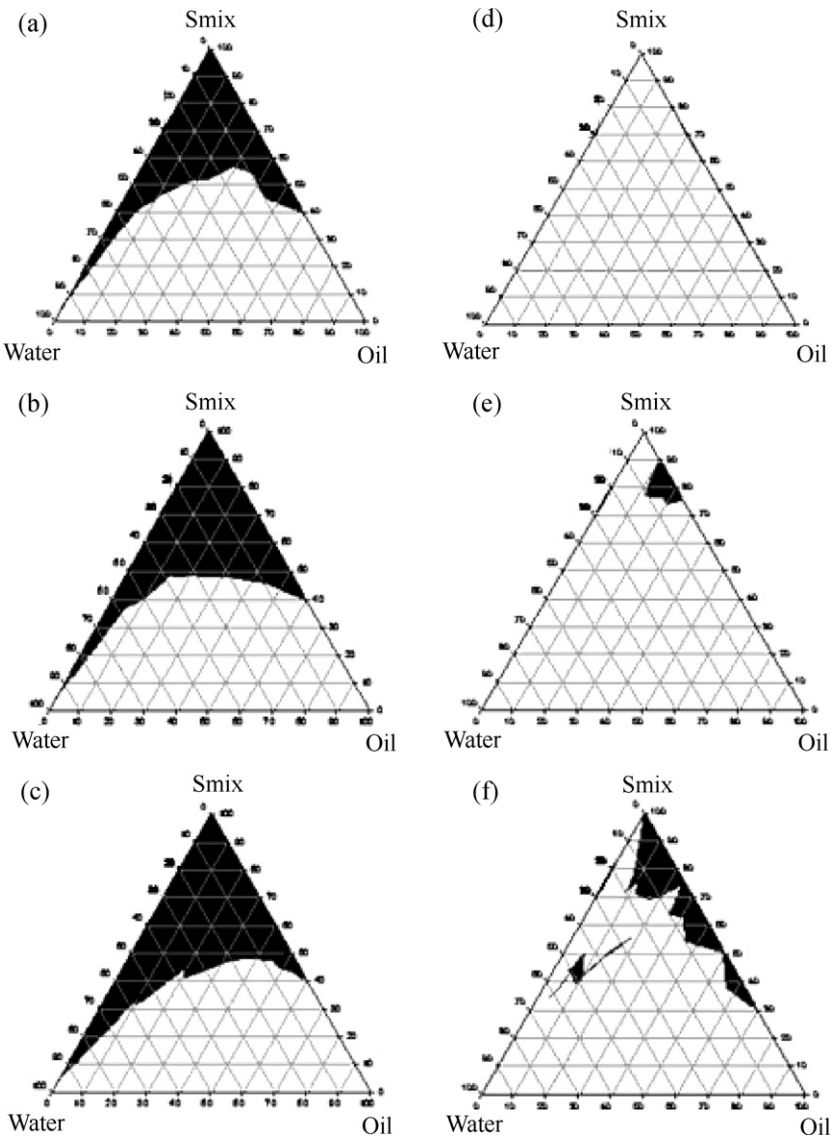

Fig. 6. Pseudoternary phase diagrams of *C. citratus* oil using surfactant system of Tween 20: ethanol with a weight ratio of 1:2 (a), 1:1 (b), and 2:1 (c) and Tween 20: hexanol of 1:2 (d), 1:1 (e), and 2:1 (f). The gray area represents the region of microemulsion.

which adversely affects its surfactant activity, therefore requiring higher concentrations of surfactant to yield stable microemulsions. The pH of the buffer had no significant effect on the phase diagrams (results not shown). This is in line with expectations and previous papers (Lawrence and Rees, 2000; Mo et al., 2000), since non-ionic surfactants were used to stabilize the emulsions.

3.2.4. Particle size measurements

The microemulsion formulations selected for the size and zeta potential analysis were composed of 10% of *C. citratus* oil, 40% of water and 50% of Smix (surfactant:ethanol = 2:1), using Brij 97, Triton X-114 and Tween 20 as surfactant. But in the system of Tween 85, 80% of Smix was used because 50% was not enough to form microemulsions. Photon correlation spectroscopy analysis showed that the particles stabilized by Brij 97, Tween 20, Triton X-114 and Tween 85 were 9.5 ± 0.1, 91.9 ± 4.4, 11.4 ± 0.1 and 78.6 ± 3.0 nm, respectively This means that indeed microemulsions were formed because their sizes range from 5 to 100 nm (Mo et al., 2000; Sintov and Shapiro, 2004). The particle size distributions were intermediate (PDI < 0.3). Of the investigated surfactants, Brij 97 and Triton X-114 gave the smallest emulsified droplets. There is however no relation between the particle size of the emulsified droplets and the physico-chemical characteristics of the used surfactants.

3.2.5. Electrical conductivity

Electrical conductivity is frequently used to investigate structural changes in oil/water/surfactant systems (Kumar and Mittal, 1999; Bauduin et al., 2005). As the data of Fig. 7 shows that low concentration of salt had no effect on microemulsions composed of water/*C.citratus* oil/Tween 20/ethanol mixture, 0.1 M NaCl solution was hence used as an aqueous phase. The relationship between aqueous phase ratio and conductivity values is shown in Fig. 8. As long as aqueous phase ratio was <0.2, the conductivity value was very low. This is in line with expectations since at low water volume fractions, the oil forms the continuous phase. However, the conductivity values substantially increased at an aqueous phase ratio above 0.2 and substantially increased when the aqueous phase volume fraction was >0.5 which points to a transition from a w/o emulsion to an o/w microemulsion (Boonme et al., 2006; Baker et al., 1984).

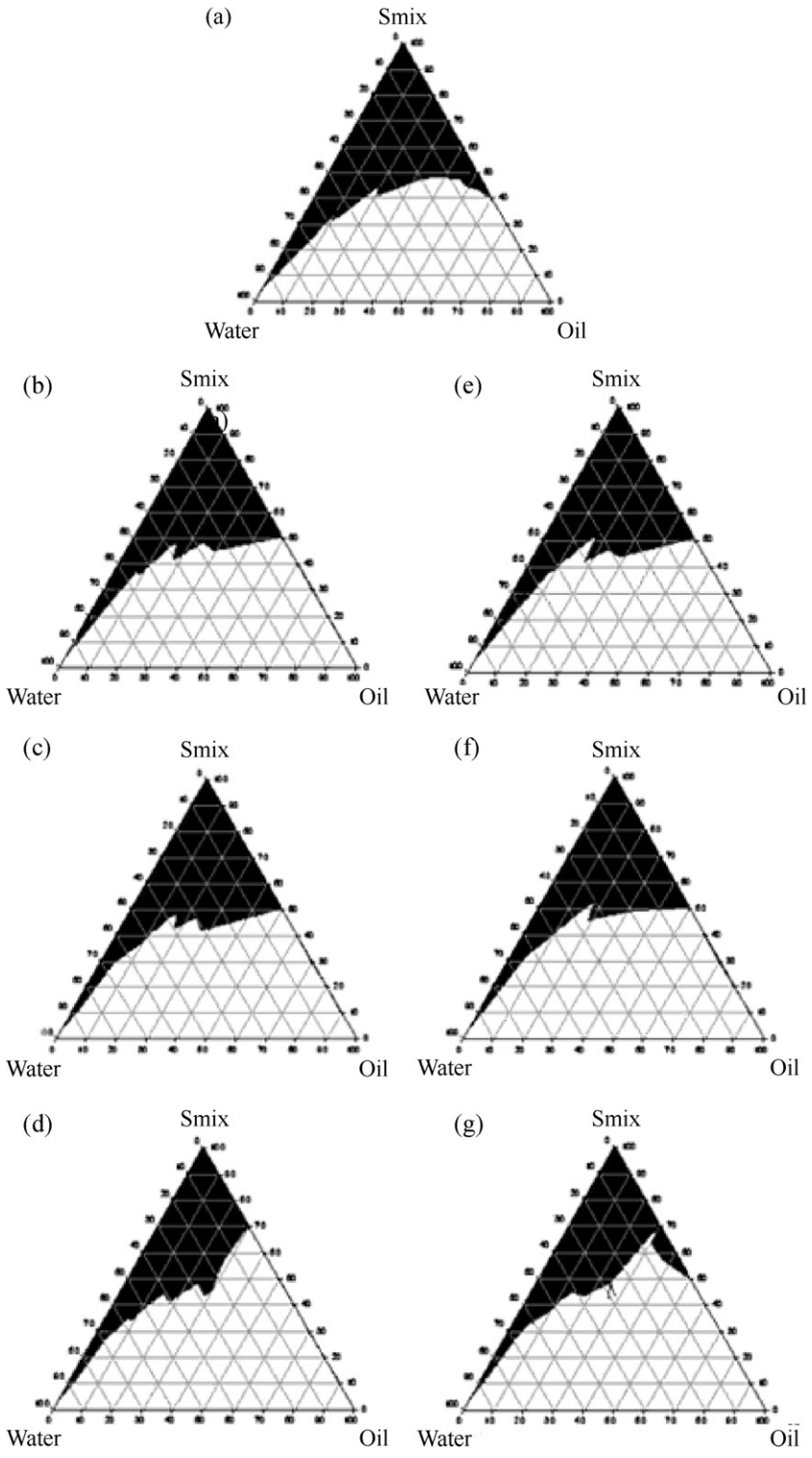

Fig. 7. Pseudoternary phase diagrams of *C. citratus* oil using 2:1 mixture of Tween 20:ethanol as surfactant system with an aqueous phase of water (a), 0.1 M NaCl (b), 0.5 M NaCl (c), 1.0 M NaCl (d), 0.1 M $CaCl_2$ (e), 0.5 M $CaCl_2$ (f) and 1.0 M $CaCl_2$ (g). The gray area represents the microemulsion region.

3.2.6. Inhibition of cholinesterase activity by microemulsion

The inhibitory activities of *C. citratus* oil alone compared with *C. citratus* oil in microemulsion formulation are shown in Fig. 9. At equal concentrations (0.1 mg/ml) of *C. citratus* oil, the inhibitory activities of microemulsion were significantly higher than that of *C. citratus* oil alone ($P < 0.05$). The inhibition on AChE and BChE of the microemulsion comprising of 10% of *C. citratus* oil, 40% of water and 50% of Smix were $69.0 \pm 3.9\%$ and $98.6 \pm 0.7\%$, respectively. The enhanced *in vitro* cholinesterase inhibitory activity of *C. citratus* oil in microemulsion formulation shows a remarkable increasing tendency when compared with the native oil.

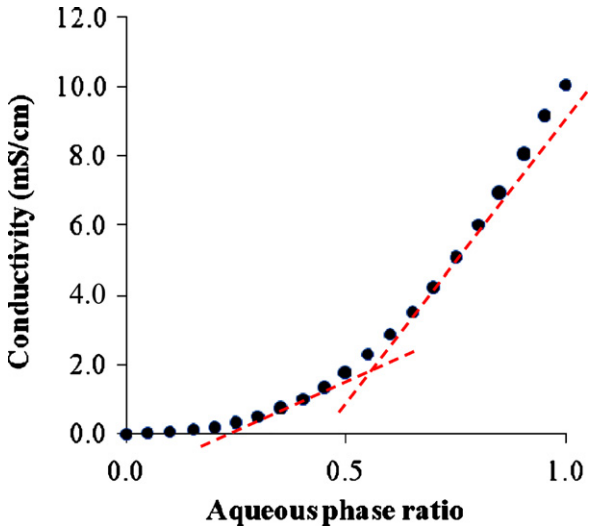

Fig. 8. Conductivity versus aqueous phase ratio of formulations containing *C. citrtus* oil/Smix = 1:9 in which Smix is a mixture of Tween 20 and ethanol (2:1).

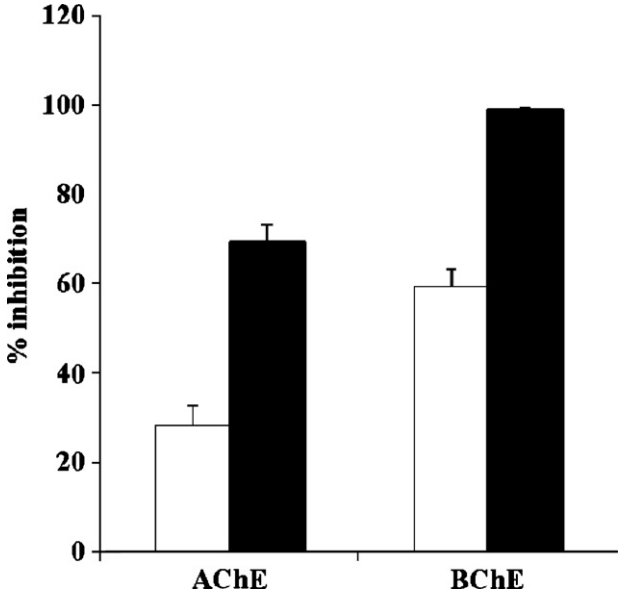

Fig. 9. Inhibitory activities of *C. citratus* oil (□) and microemulsion containing 10% *C. citratus* oil (■) against AChE and BChE.

4. Conclusion

This paper shows that essential oil of *C. citratus* with citral as main constituent, exhibited strong inhibitory effects on BChE with an IC_{50} value of 0.34 ± 0.07 μl/ml. Microemulsions of this oil with average dropet sizes ranging from ~9 to 90 nm were readily obtained using different non-ionic surfactants of which Tween 20 was selected for more in depth studies. In the system comprising Tween 20 as surfactant, ethanol was found to be a more suitable co-surfactant than hexanol to form a large microemulsion region in the pseudoternary phase diagram. The phase behavior of the water/*C.citratus* oil/Tween 20/ethanol mixture was hardly influenced by both the pH and the ionic strength of the aqueous phase. In the system containing 10% *C. citratus* oil, phase inversion occurred at a water volume fraction >0.5. This study shows that a microemulsion based on water/*C.citratus* oil/Tween 20/ethanol exhibits significantly greater inhibitory activities against both AChE and BChE when compared with the native oil. Therefore, citrtus oil loaded microemusions are attractive systems for further in vivo studies in animal models with Alzheimer's disease.

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