Original Article

Enhancement of anti-cholinesterase activity of *Zingiber cassumunar* essential oil using a microemulsion technique

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ABSTRACT: The aim of the present study was to enhance the cholinesterase inhibitory activity of *Zingiber cassumunar* (ZC) oil using a microemulsion (ME) technique. Pseudoternary phase diagrams of the oil, water, and surfactant/co-surfactant mixture were constructed using a water titration method. Effects of co-surfactant, surfactant/co-surfactant ratio, ionic strength, and pH were examined by means of the microemulsion region which existed in the phase diagrams. The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were tested by Ellman's colorimetric assay. It was found that ZC oil possesses inhibitory activity against not only AChE but also BChE. Formulation of ZC oil as ME revealed that alkyl chain length and number of hydroxyl groups of co-surfactant exhibited a remarkable effect on the pseudoternary phase diagram. Longer alkyl chains and more hydroxyl groups gave smaller regions of MEs. Ionic strength also affected the ME region. However, the phase behavior was hardly influenced by pH. The suitable ZC oil ME was composed of Triton X-114 in combination with propylene glycol. The anti-cholinesterase activity of this ME was much higher than that of native ZC oil. It exhibited twenty times and twenty five times higher inhibitory activity against AChE and BChE, respectively. ZC oil loaded ME is an attractive formulation for further characterization and an *in vivo* study in an animal model with Alzheimer's disease.

Keywords: Alzheimer's disease, acetylcholinestrase, butyrylcholinesterase, phase diagram

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder which is the most common of all brain degenerations (*1*). Patients with AD will generally present a gradual onset and a progressive sequential decline in cognitive, behavioral and motor functions which interfere with an individual's daily function and quality of life and eventually lead to an enormous burden on the country's health care system (*2-4*). Advances in preventing AD or delaying progression would have a huge global public health impact. However, there is still no specific biological marker to diagnose this disease. The definite diagnosis of AD is made upon histological verification, established by biopsy or at autopsy, of extracellular amyloid plaques and intracellular neurofibrillary tangles (*5*). Increased levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) have also been found in postmortem brain samples leading to the hypothesis that the cognitive decline in AD patients is related to progressive cholinergic degeneration (*6,7*). Various treatment strategies aimed at boosting cholinergic function, especially by the inhibition of synaptic acetylcholine degradation, has been an area of focus (*8*). Recently, a few synthetic medicines, *e.g.* tacrine, donepezil, and the natural product-based rivastigmine and galantamine have been used for treatment of cognitive dysfunction and memory loss associated with AD (*9,10*). Nevertheless, none of them can stop the disease and these compounds have been reported to have their own adverse effects including gastrointestinal disturbances and problems associated with bioavailability, which necessitates interest in finding better AChE inhibitors from natural resources and a better way to deliver these compounds.

The Zingiberaceae is among the plant families which are widely distributed throughout the tropics, particularly in Southeast Asia. Most members of this family are easily recognized by the characteristic aromatic leaves and fleshy rhizome when both of them are crushed (*11*). Several species of Zingiberaceae are used as spices, medicines and flavoring agents. *Zingiber cassumunar* Roxb. (ZC) is used in folklore remedies as a single plant or as a component of herbal recipes for the treatment

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of various conditions including inflammation, sprains, rheumatism, muscular pain, wounds, and asthma (*12*). It is also used as a mosquito repellant, an anti-dysenteric agent, a carminative, a mild laxative, and a cleansing solution for skin diseases (*13*). A number of pure compounds isolated from ZC have been shown to possess anti-inflammatory and antioxidant activity (*11,12*) which has become another focus of new treatment strategies against AD aside from anti-cholinesterase activity (*14-16*). Inflammation of the brain has been proposed to lead to neurological diseases, especially AD, since throughout the process of inflammation, the generation of neuronal cells of the adult brain is affected (*17*). Epidemiological studies point that the risk of AD is reduced among users of anti-inflammatory drugs (*18*). In the past decade, oxidative stress has also been described in the pathological changes that occur in AD (*19,20*). Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues (*21*). NO, superoxide (O_2^-) and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against infections and inflammatory conditions, contributing to some pathogenesis by promoting oxidative stress, tissue injury and even neurodegenerative disease (*22*). Since the reported antioxidant and anti-inflammation activity of ZC oil is associated with AD, investigation of the anti-cholinesterase activity of ZC oil would be an interesting aspect to confirm the possibility of using ZC oil in the treatment of AD. Moreover, incorporation of ZC oil in a novel topical formulation will also be fascinating because transdermal administration is the ideal therapeutic approach for chronic neurological disorders in elderly people since it provides sustained therapeutic plasma levels of drugs, is simple to use, and may reduce systemic adverse effects (*23*). ZC oil is a complex mixture of volatile components and its constituents can degrade during storage and lead to changes in biological activities. Therefore, incorporation of ZC oil into a suitable formulation would overcome its labile property as well as its hydrophobic property disadvantage. A microemulsion (ME) is a formulation used to incorporate ZC oil because it is an isotropic colloidal system that is formed spontaneously from appropriate combinations of oil, water, and surfactant/co-surfactant mixtures (*24*). ME is optically transparent since the internal phase droplet size ranges from 5 to 200 nm (*25,26*), which is below the wavelength of visible light. The physical appearance of the ME will be the same as the native oil. Therefore, this study aims to show the anti-cholinesterase activity of ZC oil and enhance its activity by loading the oil into a suitable ME system for topical application. The factors influencing the ME region in the pseudoternary phase diagram were also investigated to include surfactant type, co-surfactant type, surfactant/co-surfactant ratio, pH, and ionic strength of the aqueous phase. A suitable system was finally derived for the anti-cholinesterase activities.

2. Materials and Methods

2.1. *Plant materials*

Zingiber cassumunar Roxb. (Zingiberaceae) was collected from Chiang Mai, Thailand during January 2009. It was authenticated and voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand.

2.2. *Chemicals and enzymes*

AChE (specific activity 425.94 U/mg) from *Electrophorus electricus*, BChE (specific activity 7.4 U/mg) from equine serum, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), propylene glycol (PG), glycerin, polyethylene glycol 400 (PEG 400), and cetyl alcohol were from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were from Fluka (Steinheim, Germany). Octylphenoxy polyethoxy ethanol (Triton X-114) was from Acros Organics (New Jersey, USA). Ethanol, propan-1-ol and propan-2-ol were analytical grade from Merck (Darmstadt, Germany).

2.3. *Extraction of the essential oil*

The fresh rhizomes of ZC were cut into small pieces and subjected to hydro-distillation for 3 h by 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.

2.4. *GC-MS analysis*

The essential oil 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 chromatography (Agilent Technologies, CA, USA) coupled to an electron impact (EI, 70 eV) HP 5973 mass selective detector (Hewlett Packard, CA, USA) and fitted with a fused silica capillary column (HP-5MS) supplied by Hewlett Packard, 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 3°C/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*-alkanes 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.

2.5. 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 using Ellman's method (27). 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 25 μL of the extracted oils in Tris-HCl buffer containing 10% methanol were mixed accordingly. 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 with a microplate reader (Bio-Rad Laboratories Ltd., Tokyo, Japan). The Tris-HCl buffer containing 10% methanol was used as negative control for cholinesterase activity evaluation of ZC oil. In the case of inhibitory activity evaluation of the ME, 25 μL of ME containing 10% of the extracted oil was added instead of the extracted oil in Tris-HCl buffer with 10% methanol. The mixture containing 10% methanol, 30% PG, and 60% Triton X-114 was used as negative control in this case. 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 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 the absence of the oils. IC_{50} values were statistically evaluated using the Graphpad/Prism program.

2.6. Construction of phase diagrams

Pseudoternary phase diagrams of the essential oils were constructed using a water titration method. The surfactant (Triton X-114) was mixed with a co-surfactant (ethanol, propan-1-ol, propan-2-ol, cetyl alcohol, glycerin, PG, or PEG 400) at a weight ratio of 2:1 to obtain the 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 ME when they appeared visually as clear liquids. Instead of water, acetate buffers of pH 4.0 and 6.0 (composed of 0.1 M acetic acid and 0.1 M sodium acetate), phosphate buffers of pH 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 (NaCl) and magnesium chloride ($MgCl_2$) 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 using the OriginPro 8 program.

3. Results and Discussion

3.1. GC-MS of ZC oil

The chemical compositions of ZC oil investigated by GC-MS are shown in Table 1. Fourteen components were identified accounting for 95.21% of the total yields. Terpinen-4-ol (67.06%) and γ-terpinene (13.26%) were major constituents. The results were in good agreement with previous reports (14,28).

3.2. Cholinesterase inhibitory activity of ZC oil

The dose response curves for anti-AChE and anti-BChE with ZC oil are shown in Figures 1 and 2, respectively. The calculated IC_{50} values show that ZC oil can be characterized as a moderate BChE inhibitor with IC_{50} of 0.355 ± 0.137 mg/mL and a weak AChE inhibitor with IC_{50} of 5.573 ± 0.176 mg/mL. Acetylcholine is a neurotransmitter released at the synaptic gap. The pathological features in central nervous system disorders are identified by neurotransmitter disturbances and are insufficient especially in cholinergic functions (10). Normally, acetylcholine is hydrolyzed by the cholinesterase enzyme. A greater amount of the enzyme leads to lower amount of aceylcholine found in the synaptic gap. Inhibition of cholinesterase therefore can restore the level of acetylcholine in the brain (29). AChE is lost up to nearly 85% in specific brain regions in AD patients, whereas the BChE level rises with disease progression (30). Distinctly, the imbalance in cholinesterase activity in the AD brain modifies the normally supportive role of BChE and represents the reason behind recent synthesis of new selective BChE inhibitors for mild to moderate forms of AD (31). Kim *et al.* (2006) have reported the memory enhancing effect of essential oil from *Abies koreana*, of which terpinen-4-ol was the major component, on scopolamine-induced amnesia in mice (32). Moreover, terpinen-4-ol has been reported for its inhibition of AChE activity (33).

Table 1. Chemical compositions of the essential oil of ZC

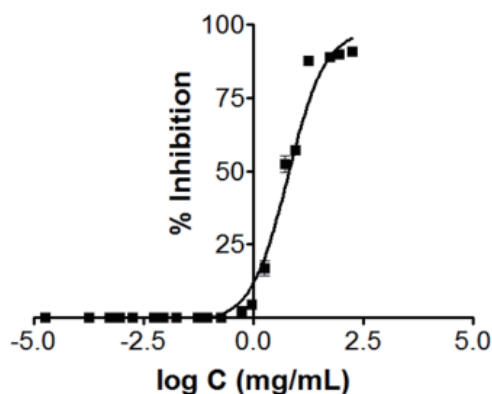| Retention time (min)	Compound	Peak area (%)
3.21	α-Terpinene	0.40
3.56	γ-Terpinene	13.26
3.68	*cis*-Sabinenehydrate	0.54
3.99	α-Terpinolene	3.04
4.16	*trans*-Linaool oxide	0.47
4.58	Neo-allo-ocimene	2.52
4.90	γ-Terpinene	1.76
5.93	Terpinen-4-ol	67.06
6.07	*p*-Cymen-8-ol	2.36
6.16	Dill ether	0.59
6.41	*cis*-Piperitol	1.03
10.42	δ-Elemene	0.31
16.44	Allo-ocimene	0.68
18.98	Caryophyllene oxide	1.19
	Total	95.21

Figure 1. Dose response curves of ZC oil against AChE. AChE was added to the mixture of 50 mM Tris-HCl buffer pH 8.0, 1.5 mM ATCI, 3 mM DTNB, and the tested oil in Tris-HCl buffer containing 10% methanol. The reaction was spectrophotometrically determined at 415 nm by a microplate reader.
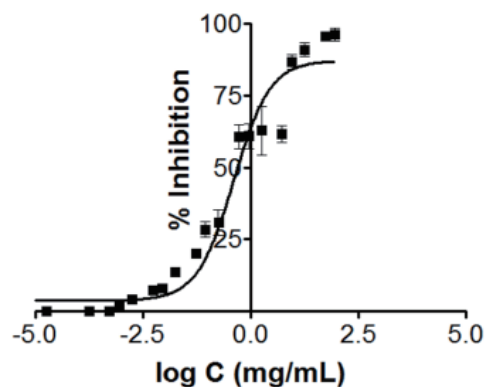

Figure 2. Dose response curves of ZC oil against BChE. BChE was added to the mixture of 50 mM Tris-HCl buffer pH 8.0, 1.5 mM ATCI, 3 mM DTNB, and the extracted oils in Tris-HCl buffer containing 10% methanol. The reaction was spectrophotometrically determined at 415 nm by a microplate reader.

Therefore, terpinen-4-ol is one of the possible affective agents that account for the anti-cholinesterase activity. However, a complex interaction between its various constituents, in which synergistic responses might be produced, resulted in the inhibitory activity of ZC oil (*34*).

3.3. Influence of co-surfactant and surfactant/co-surfactant ratio on the phase diagrams

ME is a clear, thermodynamically stable and optically isotropic system. ME forms spontaneously upon mixing a suitable proportion of oil, water, and surfactant either alone or in combination with a co-surfactant, thereby overcoming the need for any additional input of energy. Triton X-114, nonionic surfactant, was employed in this study since it shows low irritancy and low toxicity (*35*). The influence of co-surfactant type was studied since it can reduce surface tension and increase flexibility of the interface by partitioning themselves among the oil, water, and interface domains (*36*) which lead to the spontaneously formed ME (*37*). Since co-surfactant participates in a micelle and adjusts the polarity of water and oil, it thus affects the system of phase behavior and nature of the ME components (*38*). Therefore, the study of suitable co-surfactants would be appropriate. The phase behavior of the ZC oil/Triton X-114/co-surfactant/water system was represented in a pseudoternary phase diagram, in which ZC oil is one component, another one is water, and the third component is Smix, which is a mixture of Triton X-114 and co-surfactant. The ternary phase diagram was constructed adopting a simple titration method. Smix, in which the Triton X-114 and co-surfactant mass ratio was fixed, was first prepared by combining the required mass of Triton X-114 and co-surfactant. Then an appropriate quantity of ZC oil was introduced into the emulsifier and water was the titration component. The phase boundary was noted by observing

the transition from turbidity to transparency or from transparency to turbidity.

The effect of chain length of linear alcohols on the phase behavior of ME systems were investigated by using ethanol, *n*-propanol and cetyl alcohol. Figure 3 shows phase diagrams as well as percentage of ME region which occurred when various types of co-surfactant were mixed with Triton X-114. The results indicated that cetyl alcohol, the longest alkyl chain alcohol in this study, gave a very sparse ME region with Triton X-114. Moreover, the ME regions in the phase diagrams using ethanol were larger than that of *n*-propanol. These results were in accordance with the previous study of Alany *et al.* (*39*) which indicated that the ME region in the phase diagram was reduced upon increasing the chain length of alkane alcohols. Branch chained alcohols had no effect on the formation of the ME.

The effect of hydroxyl group numbers on the phase behavior was also considered. Isopropanol was selected as a representative of alkane mono-ol while PG and glycerin represented an alkane diol and triol, respectively. The results, as shown in Figure 3, indicate that glycerin was not a good co-surfactant and the ME region nearly disappeared with glycerin because of its highly hydrophilic property. On the other hand, PG and isopropanol were appropriate to form ME with Triton X-114 since they gave a larger ME area in the phase diagram. Moreover, PG showed a higher performance in ME formation when compared with PEG 400 because of its compact molecule (*35*). A likely explanation is that the complicated co-surfactant cannot form the ME properly if the structure of surfactant is complex (*39*). Therefore, PG was selected as a co-surfactant for further investigations because it gave a large ME region area in the phase diagram as well as its non-volatile properties. Figure 4 shows the effect of surfactant to co-surfactant ratio. The higher ratio of surfactant to co-surfactant gave a larger ME region in the phase diagram.
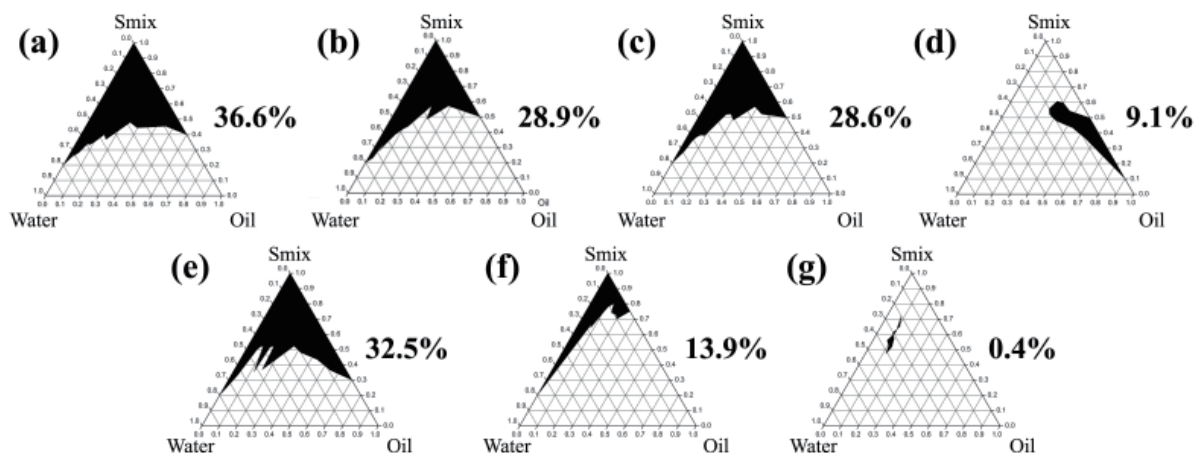
Figure 3. Pseudoternary phase diagrams of ZC oil ME showing the effect of co-surfactant types. The ZC oil ME comprises ZC oil/Triton X-114/co-surfactant/water with ethanol (**a**), propan-1-ol (**b**), propan-2-ol (**c**), cetyl alcohol (**d**), PG (**e**), PEG 400 (**f**), and glycerin (**g**) as a co-surfactant. The ratio of Triton X-114 to co-surfactant was 2:1. The dark area expressed by the number (%) represents the region of microemulsion.
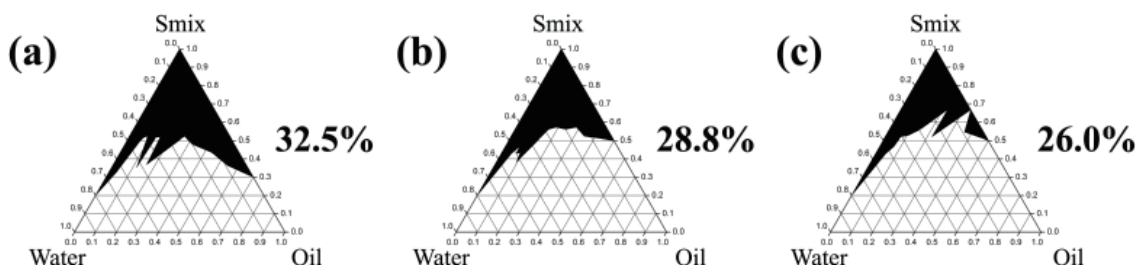

Figure 4. Pseudoternary phase diagrams of ZC oil ME showing the effect of surfactant to co-surfactant ratio. The ZC oil ME is comprised of ZC oil/Triton X-114/PG/water mixtures with a Triton X-114 to PG ratio of 2:1 (**a**), 1:1 (**b**), and 1:2 (**c**). The dark area expressed by the number (%) represents the region of microemulsion.

3.4. *Effect of ionic strength and pH on the phase diagrams*

Electrolyte was believed to have an effect on the region of MEs in the phase diagram because it affected the size of the emulsified droplets (*40*). Therefore, the effect of monovalent and divalent salts on the ZC oil ME system was investigated in the present study. Figure 5 exhibits the effect of electrolytes on the ZC oil ME system using Triton X-114 as a surfactant. NaCl was employed as a monovalent salt while $MgCl_2$ was employed as a divalent salt in this investigation. The results indicated that both electrolytes affected the ME regions. Moreover, $MgCl_2$ showed a stronger effect than NaCl. It was found that with higher concentration of salts, lower ME regions were obtained. This result was due to the salting effect of the divalent electrolyte (Mg^{2+}) that was higher than that of the monovalent electrolyte (Na^+) (*41*). In addition, the pH effects were also investigated and the results are shown in Figure 6. pH exhibited no effect on the ME region of the ZC oil system. Therefore, we concluded that the ME system comprised of ZC oil/Triton X-114/PG/water was hardly effected by variation of pH but could be affected by the ionic strength of the aqueous phase.

3.5. *Cholinesterase inhibitory activity of ZC oil/Triton X-114/PG/water*

The IC_{50} values of ZC oil/Triton X-114/PG/water system against AChE and BChE were compared with the native ZC oil alone. The results are shown in Table 2. It was found that ZC oil in the ME system possessed about twenty times higher inhibition of AChE and about twenty five times higher inhibition of BChE. This result could be due to the increase in solubility of the oil by the ME technique which were more compatible with the enzymes in the test medium.

4. Conclusion

ZC oil possesses inhibitory activity against not only AChE but also BChE. Formulation of ZC oil as ME revealed that alkyl chain length and number of hydroxyl groups of co-surfactants exhibited a remarkable effect on the pseudoternary phase diagram. Longer alkyl chains and more hydroxyl groups gave smaller regions of MEs. Ionic strength also affected the ME region. However, the phase behavior was hardly influenced by pH. The most
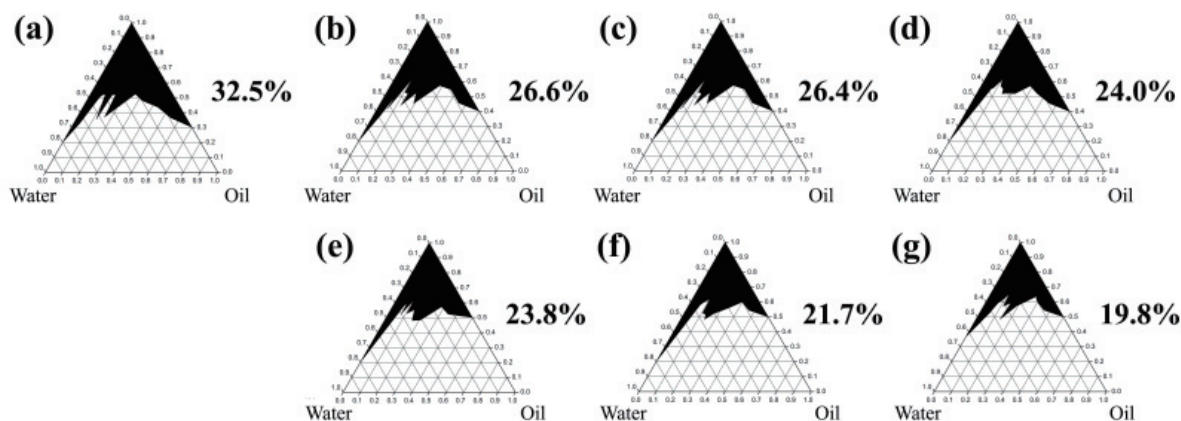
Figure 5. Pseudoternary phase diagrams of ZC oil ME showing the effect of ionic strength. The ZC oil ME using a 2:1 mixture of Triton X-114:PG as surfactant system contains an aqueous phase of water (**a**), 0.1 M NaCl (**b**), 0.5 M NaCl (**c**), 1.0 M NaCl (**d**), 0.1 M MgCl$_2$ (**e**), 0.5 M MgCl$_2$ (**f**) and 1.0 M MgCl$_2$ (**g**). The dark area expressed by the number (%) represents the microemulsion region.
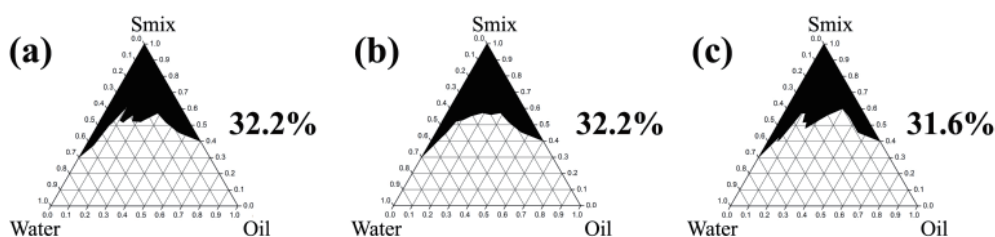

Figure 6. Pseudoternary phase diagrams of ZC oil ME showing the effect of pH. The ZC oil ME using a 2:1 mixture of Triton X-114:PG as surfactant system contains an aqueous phase of water pH 4.0 (**a**), 6.0 (**b**) and 8.0 (**c**). The dark area expressed by the number (%) represents the microemulsion region.

Table 2. IC$_{50}$ of ME comprised of ZC oil against AChE and BChE

Formulations	IC$_{50}$ (mg/mL)	
	AChE	BChE
ZC oil	5.573 ± 0.176	0.355 ± 0.137
ZC oil/ Triton X-114/PG/water	0.252 ± 0.096	0.014 ± 0.002

suitable ZC oil ME was composed of Triton X-114 in combination with PG. The anti-cholinesterase activity of this ME was much higher than that of native ZC oil. It exhibited twenty times and twenty five times higher inhibitory activity against AChE and BChE, respectively. A ZC oil loaded ME is an attractive formulation for further characterization and *in vivo* studies in animal models with AD.

Acknowledgements

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