Original Article

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Nanoemulsion: A suitable nanodelivery system of clove oil for anesthetizing Nile tilapia

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Summary **Clove oil ethanolic solution (CL-EtOH) have always been used for fish anesthesia. However, ethanol causes major side effect of fish hypersensivity. In this study, clove oil loaded nanoemulsion (CLN) was developed in order to enhance water miscibility of clove oil without using ethanol in the preparations. The obtained CLN was characterized in terms of droplet size, size distribution expressed as polydispersity index (PDI), and zeta potential. The anesthetic effect of CLN in comparison with CL-EtOH on *Oreochromis niloticus* (Nile tilapia) was investigated. The results showed that the best CLN was composed of 20% w/w clove oil and 15% w/w polysorbate 20. This CLN has internal droplet size of 63.2 ± 1.0 nm, PDI of 0.31 ± 0.04, and zeta potential of – 30.3 ± 8.1 mV. GC-MS analysis indicated that eugenol was the main compound in clove oil. It was found that the induction time to anesthesia for Nile tilapia that received this CLN was shorter than that received CL-EtOH at the same eugenol concentration. The results of this study showed the potential of nanoemulsion on water miscible and efficacy enhancing of clove oil without using ethanol. The obtained CLN from this study is a promising formulation for fish aquaculture where fish sedation is required.**

Keywords: Nanoemulsion, clove oil, fish anesthesia, Nile tilapia, *Oreochromis niloticus*

1. Introduction

Fish anesthesia is an important step before fish handling in aquaculture and veterinary research fields in order to ease handling and minimize stress and injury of fish (*1*). Ideal anesthetics for fish should have suitable properties such as water miscibility, convenience for use, effectiveness with low concentration, low physiological perturbation, and low cost, as well as safety for the fish, human, and environment (*2*). Chemical synthetic anesthetics are generally more expensive and toxic to environment than natural agents such as plant extracts. The fish exposed to chemical anesthetics require a long withdrawal period before human consumption (*3*). Clove oil has been used as food additive for human for a long time. It shows anesthetic activity to various kinds of fish species according to its bioactive compound, eugenol (*4-6*). This compound can depress medullary respiratory centers and reduce the gill ventilation with hypoxia leading to bradycardia and decrease blood flow in the gills (*7*). Clove oil can be easily obtained by hydrodistillation of buds of *Syzygium aromaticum*, therefore clove oil is characterized as natural anesthetic. It is also inexpensive compared with the chemical synthetic anesthetic agents. Clove oil is regarded as safe for both fish and human (*8*). Using clove oil for fish anesthesia does not require any withdrawal period for human consumption (*9*). However, clove oil is immiscible with water. Ethanol has always been used when diluting clove oil in water or in aqueous preparations in order to enhance water miscibility of clove oil. Clove oil ethanolic solution (CL-EtOH) is therefore available nowadays. However, hyperactivity of the fish was reported during anesthetizing with CL-EtOH (*10,11*). To avoid this side effect, the anesthetic preparations of clove oil without ethanol or any organic

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solvents are attempted to be developed.

Nanoemulsion is usually a dispersion of oil droplets in the aqueous system. Currently, nanoemulsion plays an important role on improving the properties of many hydrophobic active compounds for human and animals particularly on increasing water miscibility of those active compounds (*12*). The advantage of nanoemulsion is that it possesses high kinetic stability due to its extremely small droplet size of the internal phase, approximately 20-200 nm (*13*). This nanoformulation can be stable against creaming or sedimentation or phase separation. In this study, clove oil loaded nanoemulsions (CLN) were mechanically prepared using high-shear stirring and high-pressure homogenization in order to enhance water miscible of clove oil. The obtained CLN were characterized and tested for anesthetic effect in comparison with CL-EtOH using *Oreochromis niloticus* (Nile tilapia) as a fish model.

2. Materials and Methods

2.1. *Materials*

Clove oil was purchased from Thai-China Flavours & Fragrances Industry (Nonthaburi, Thailand). Polysorbate 20 and polysorbate 80 were from Sigma-Aldrich (Darmstadt, Germany). Dichloromethane was from (Merck Millipore, Germany). Ethanol was from Emsure (Darmstadt, Germany). Nile tilapia were purchased from a local farm in Chiang Mai, Thailand. The fish were allowed to acclimate at laboratory conditions for 2 weeks.

2.2. *Chemical analysis of clove oil*

Analysis of clove oil was performed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 gas chromatograph coupled to electron impact (EI, 70 eV) using a Hewlett Packard (HP) mass selective detector (MSD), model HP 5973-MSD (Agilent Technologies Inc, USA). The fused silica capillary column (HP5-MSI; 30.0 m × 0.25 mm *i.d.* × 0.25 µm film thickness) was used. The analytical conditions were as follows; carrier gas: helium (1.0 mL min^{-1}), injector temperature: 25°C, oven temperature: 3 min isothermal at 70°C, increased from 3°C min^{-1} to 188°C then from 20°C min^{-1} to 280°C and 3 min isothermal at 280°C. The detector temperature was 280°C. The oil sample was diluted with dichloromethane to 1:100 volume ratio. An exact portion of 1 µL of the diluted oil sample was injected. The identification of individual compound was based on their retention times relative to those of authentic samples and matching spectral peaks available in Wiley, NIST, and NBS mass spectral libraries.

2.3. *Preparation and characterization of CLN*

Two formulations of CLN (CLN-10 and CLN-20) composed of 10% and 20% w/w clove oil, respectively, were prepared. Polysorbate 20 and polysorbate 80 were used as surfactants for these formulations. The surfactant was firstly added to the aqueous phase to reach a final concentration in CLN of 15%. Then the oil phase was dispersed in the aqueous phase with stirring at 40-50°C. The mixture was emulsified by high shear homogenizer (Ultra-Turrax T25, IKA-WERKE, Germany) at 16,000 rpm for 3 min. Subsequently, this pre-emulsion was passed through a high pressure homogenizer (EmulsiFlex-C3, Avestin, Canada) for 10 cycles at the pressure of 1,000 bars. The homogenized nanoemulsions were cooled down to room temperature (about 30°C). The CLN formulations obtained were evaluated for droplet size, size distribution, and zeta potential by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nano-ZS, Malvern, UK) once after preparation and at day 30 after storage. During storage, the physical appearance of the formulations was observed. The best CLN was selected for evaluation of anesthetic efficacy in Nile tilapia.

2.4. *Effect of CLN on fish anesthesia*

Samples of Nile tilapia with 42.85 ± 2.67 g body weight and 14.51 ± 0.54 cm length were randomly collected from the holding tanks (n = 20). The fish were fed twice daily with a commercial dry feed (INTEQC Feed, Thailand) and held in natural light conditions. These experimental methods were approved by the Animal Care Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC no. R19/2555). The content of eugenol in each formulation was calculated based on the percentage of eugenol in the clove oil obtained from the GC-MS results. An exact amount of the selected CLN was added to the induction tank (10 × 10 × 15.5 cm) to have a final eugenol concentration of 40 mg/L after adjusting the volume with water to 1 L. To a positive control tank, an exact amount of CL-EtOH having 1:9 volume ratio of eugenol to ethanol was added to have the same eugenol concentration as CLN after adjusting the volume with water to 1 L. Nile tilapia was individually transferred into these tanks (3 fish/tank). The effects of CLN or CL-EtOH on fish anesthesia were investigated by determining the induction time to anesthesia and recovery time from anesthesia. After reaching the desired stage of anesthesia, the fish were transferred into a recovery tank (15.5 × 25.5 × 18 cm) containing 5 L of oxygenated water. The fish behavior or mortality was observed until they are fully recovered to the desired recovery stage.

2.5. *Statistical analysis*

All experiments were done in triplicate. The data were presented as mean ± SD. Statistical evaluation of anesthesia induction and recovery times in Nile tilapia

was performed by *t*-test where $p < 0.05$ was considered to indicate the significant differences.

3. Results

3.1. *Chemical analysis of clove oil*

Clove oil appeared as a clear pale yellowish liquid. GC-MS chromatogram of clove oil showed the presence of 3 identifiable components (Table 1) which represented 98.08% of the total oil. It was found that the oil consisted of eugenol as a major component (96.11%). Caryophyllene and naphthalene were found as minor components.

3.2. *Preparation and characterization of CLN*

According to the difference of surfactants, polysorbate 20 and polysorbate 80, and of clove oil concentrations, 10% and 20%, used in the preparation of CLN, therefore 4 CLN formulations were obtained. It was found that CLN-10 and CLN-20 containing polysorbate 20 presented good appearance without any changes whereas those containing polysorbate 80 showed phase separation within 24 h. Therefore, only CLN formulations containing polysorbate 20 were selected for further investigation on droplet characteristics. The results were shown in Table 2. It was found that CLN-20 possessed significantly smaller droplet size than CLN-10. Both formulations presented narrow size distribution, expressed as polydispersity index (PDI) values. The size distributions of CLN-10 and CLN-20 were confirmed by the distribution curves shown in Figure 1 and Figure 2, respectively. From these figures, the peak intensity of CLN-10 was 98.2% and that of CLN-20 was 97.4%. Zeta potential of both CLN formulations was less than –30 mV. Keeping the formulations in room temperature (about 30°C) for 3 days, the appearance of phase separation occurred in CLN-10 whereas no phase separation was observed in CLN-20 even keeping for 30 days. CLN-20 containing polysorbate 20 therefore was concluded to be the best formulation suitable for further study because it demonstrated the highest stability, smallest droplet size, lowest PDI, and the optimum zeta potential.

3.3. *Effect of CLN on fish anesthesia*

In this experiment, CLN-20 containing polysorbate 20 was selected to compare the anesthetic effects with CL-EtOH on Nile tilapia. The induction to anesthesia in fish was divided into various stages according to the depth of anesthesia (*14*) as shown in Table 3. Stage 4 or a final stage of anesthesia was confirmed by checking pain reflex at a tail near a fish caudal fin. The fish recovery from anesthesia was also divided into various stages according to the level of recovery (*15*), as shown in Table 4. The effects of CLN in comparison with CL-EtOH on anesthesia of Nile tilapia was shown in

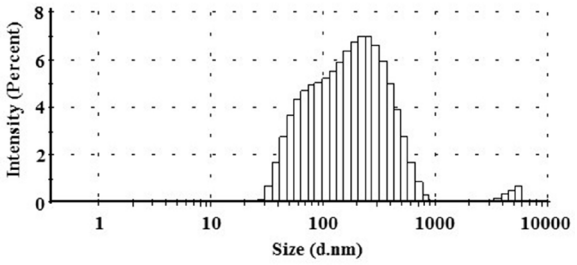

Figure 1. PCS analysis of CLN-10.

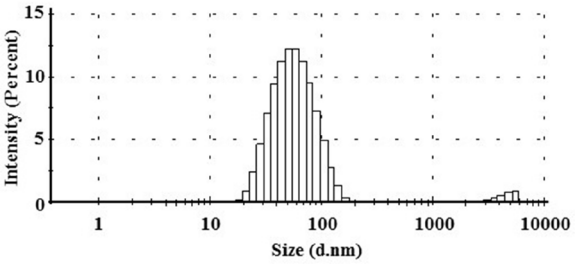

Figure 2. PCS analysis of CLN-20.

Table 1. GC-MS analysis of clove oil

Components	Retention time (min)	Area (%)
Eugenol	19.96	96.11
Caryophyllene	21.59	1.34
Naphthalene	22.90	0.63

Table 2. Internal droplet characterization of CLN by PCS

Characteristics	Formulations	
	CLN-10	CLN-20
Size (nm)	222.4 ± 9.8	63.2 ± 1.0
PDI	0.34 ± 0.08	0.31 ± 0.04
Zeta potential (mV)	–31.0 ± 3.3	–30.3 ± 8.1

Table 3. Stages of induction of fish anesthesia

Stages	Description of anesthesia
2	Partial loss of equilibrium
3a	Total loss of equilibrium but retain swimming ability
3b	Swimming ability stops
4	No responds to pressure on the caudal peduncle

Table 4. Stages of recovery from fish anesthesia

Stages	Description of recovery
1	Body immobilized but opercular movements just starting
2	Regular opercular movements and gross body movements beginning
3	Fish have normal equilibrium and normal swimming ability

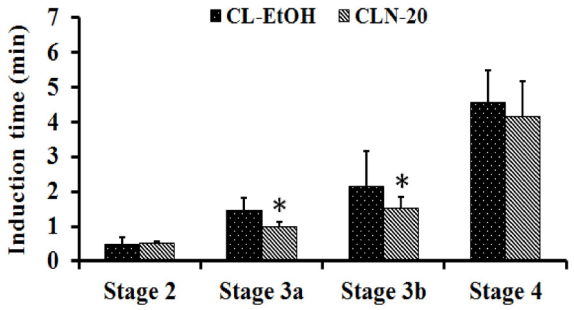

Figure 3. Times required for induction to each stage of anesthesia in Nile tilapia (n = 20) after exposure to CLN-20 and CL-EtOH. Asterisk (*) represents a significant difference ($p < 0.05$).

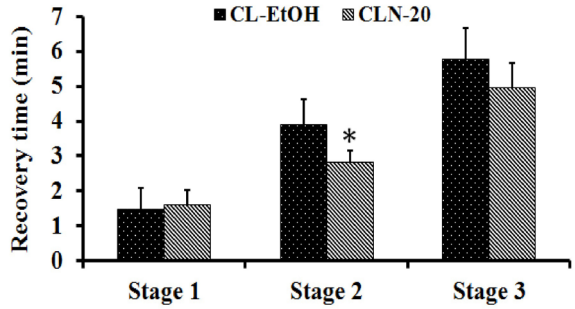

Figure 4. Times required for each stage of recovery from anesthesia in the anesthetized Nile tilapia (n = 20) caused by CLN-20 and CL-EtOH. Asterisk (*) represents a significant difference ($p < 0.05$).

Figure 3. CLN caused the fish to enter stage 3a and 3b of anesthesia within 1.0 ± 0.1 and 1.5 ± 0.3 min, respectively, which was significantly faster than CL-EtOH. The fish anesthetized with CLN-20 reached stage 2 of recovery from anesthesia within 2.8 ± 0.3 min, significantly shorter than those anesthetized with CL-EtOH, as shown in Figure 4.

4. Discussion

The common form of emulsion always shows physical instability such as aggregation of internal oil droplets or phase separation upon storage for a period of time. According to this, nanoemulsion is developed in the current study because it is much stable than the common emulsion. Nanoemulsion can be formulated by using suitable surfactant. Even there are many types of surfactant, the nonionic type such as polysorbates are the most popular for pharmaceutical preparations because of their compatibility with various active compounds. In the systems containing oil and water, nonionic surfactants can rapidly adsorb at oil/water interface to reduce the oil/water interfacial tension and provide steric or electrostatic hindrance to the dispersed internal droplets to prevent droplet aggregation (*16*). Previous literature has reported the developed nanoemulsions of clove oil using polysorbate 80 for enhancing antibacterial activity of clove oil (*17*). However, their formulations contained low amount of clove oil and used high concentration of surfactant. In the present study, polysorbate 20 and polysorbate 80 were compared for forming CLN with high loading of clove oil. The hydrophilic-lipophilic balance (HLB) of polysorbate 20 and polysorbate 80 are obviously different so that it is interesting to use both of them in the current study in order to investigate the effects of the HLB on loading efficiency of clove oil. The results demonstrate that those CLN containing polysorbate 20 have higher efficiency on loading clove oil than those containing polysorbate 80 indicating the effects of HLB on loading this oil in the nanoemulsions. Interestingly, CLN-20 which the concentration of clove oil is 2 times higher than CLN-10 showed better characteristics and higher stability than CLN-10. This result indicates that to prepare the best nanoemulsion, the ratio of oil, surfactant, and water should be in optimum. Excess surfactant can lead to a phase separation. Low PDI values indicate monodispersed systems (*18*) and can be related to high stability on storage for nanoemulsions (*19*). CLN-20 possesses significantly smaller droplet size and lower PDI values than CLN-10, therefore the stability of CLN-20 is higher than CLN-10. The zeta potential indicates the degree of electrostatic repulsion between particles in a dispersion. A high zeta potential value provides high stability to the dispersion and prevents aggregation (*20*). The high zeta potential in CLN is considered to be due to the interaction between the hydrophilic parts of the surfactant and the water molecules during the process of nanoemulsion formulation to form potentially negative charges. These negative charges can be absorbed to the emulsifier layer of oil/water interface and electric double layer similar to that of ionic form (*21,22*).

The efficacy of clove oil on anesthesia of Nile tilapia has been previously evaluated (*23,24*). However, those studies evaluated clove oil or synthetic eugenol in ethanolic solutions. The present study showed that clove oil loading nanoformulation like CLN is much better than the ethanolic solution like CL-EtOH. Besides no side effect of ethanol, CLN can cause faster induction of fish anesthesia. Nanoformulations can enhance the water miscibility of insoluble drugs (*25,26*). Due to the extremely small size with large interfacial area of internal oil droplets surrounding with the suitable surfactant in CLN, the miscibility of clove oil in aqueous systems can be enhanced. This leads to the rapid absorption of clove oil via the fish gills and skin, depression at medullary respiratory centers as a consequence, and efficient delivery of the active eugenol (in clove oil) to the sites of action with rapid onset. In conclusion, nanoemulsions can improve the aqueous miscibility and efficacy of clove oil suitable utility for fish anesthesia. CLN composed of 20% w/w of clove oil and 15% w/w of polysorbate 20 is the best formulation for this purpose.

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