Kasetsart J. (Nat. Sci.) 43 : 132 - 140 (2009)

Efficacy of Synthetic Eugenol as an Anesthetic for Nile Tilapia (*Oreochromis niloticus* Linn.)

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ABSTRACT

The efficacy of synthetic eugenol as an anesthetic for Nile tilapia (*Oreochromis niloticus* Linn.) fry was investigated. An acute toxicity test and the efficacy of synthetic eugenol as an anesthetic were studied and compared with clove oil-derived eugenol and MS-222 under similar conditions. The acute toxicity test indicated that the 24-hr LC_{50} value of synthetic eugenol, clove oil-derived eugenol and MS-222 was 16.98, 16.95 and 72.5 ppm, respectively. The efficacy test, involving 20 min exposure to various concentrations of the three anesthetics indicated that synthetic and clove oil-derived eugenol caused sedation at 5 ppm. A dose of 20 ppm of synthetic eugenol caused the loss of reflex reactivity (stage 5 of anesthesia) with the induction time (3.40 min) slightly over the limit of 3 min, while it took 2.86 min for clove oil-derived eugenol. A higher dose of MS-222 was required than for the two other anesthetics, with 30 ppm necessary to induce the sedation stage and 120 ppm to induce stage 5 anesthesia within 2.16 min. However, this concentration caused 50% mortality after 20 min of exposure. The recovery time from anesthesia for fish exposed to each anesthetic was prolonged according to the higher dose exposure of each anesthetic. The results of this experiment clearly indicated that synthetic eugenol could be an effective anesthetic for handling and transport purposes of this species judging from the concentration for the induction of various stages of anesthesia, recovery time and safety for tilapia fry.

Key words: Tilapia (*Oreochromis niloticus*), anesthetic, synthetic eugenol, clove oil-derived eugenol, MS-222

INTRODUCTION

Nile tilapia (*Oreochromis niloticus* Linn.) is an important freshwater fish, which has been cultured in every part of Thailand and has quickly expanded because of its high demand in local and foreign markets. Tilapia fish culture is commonly exposed to handling and transportation that can cause negative effects on the physiology and behavior of the fish due to stress. Generally, fish transportation is conducted in crowded conditions, resulting in the deterioration of fish health after transport. The use of anesthetics during fish transportation can prevent physical injury and reduce metabolism (less oxygen consumption and excretion) by reducing or blocking activation of the hypothalamo–pituitary–interrenal (HPI) axis associated with the release of cortisol. Cortisol can cause various physiological responses to overcome or compensate for the stress, such as suppression

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of immune system responses (Small, 2003).

Currently, there are many anesthetics available for aquatic animals, such as metomidate, 2-phenoxyethanol, quinaldine, tricaine methanesulfonate (MS-222), benzocaine, clove oil and Aqui-S^{TM} (Pirhonen and Schreck, 2003; Small, 2003; Iversen *et al*., 2003; Coyle *et al*., 2004). However, most are not approved by the Food and Drug Administration of the United States of America (USFDA) as an anesthetic for fish grown for human consumption because of potential carcinogenic and mutagenic effects that may be harmful to humans. MS-222 is the only anesthetic that is approved by USFDA, but it has low efficacy on plasma cortisol control and it is expensive (Coyle *et al*., 2004). An option as an alternative anesthetic is eugenol (2-methoxy-4-(2-propenyl) phenol), which is a derivative substance in clove oil that is distilled from the clove tree (*Eugenia aromatica*). Eugenol is listed in the USFDA category of materials as "generally regarded as safe" because it has the characteristics of an ideal anesthetic, such as low cost, no withdrawal period, lack of negative effects on fish feeding and decreasing blood cortisol concentrations. Moreover, it has been reported that it could prevent the stress-induced decrease of the neutrophil function, in contrast to MS-222 (Pirhonen and Schreck, 2003; Small, 2003; Palic *et al*., 2006). Consequently, it should be considered as a potential future anesthetic in aquaculture.

Eugenol for use with aquatic animals is usually obtained by an extraction method, which contains about 70-99% of active ingredient by weight. However, the eugenol used in this study was synthetically derived, which is easy to prepare and has 100% active ingredient. The purposes of this study were to determine the effectiveness of synthetic eugenol as an anesthetic by comparing it with two other anesthetics: clove oil (99% eugenol by weight) and MS-222. The results from this study will be used to determine the potential application of synthetic eugenol as an anesthetic for use in tilapia handling and transportation.

MATERIALS AND METHODS

Experimental animals

Healthy Nile tilapia (*Oreochromis niloticus* Linn.) fry with an average weight of 3.0±0.09 g (mean ± standard error, SE) and an average length of 2.63±0.25 cm were used in the experiment. Fish were acclimated and fed with commercial pellet feed for three days in a 1,000 litre fiberglass tank prior to the experiment.

Anesthetics

Synthetic eugenol (100% eugenol by weight), clove oil (99% eugenol by weight) and MS-222 were used as fish anesthetics in this study. Synthetic eugenol and clove oil were obtained from the Faculty of Pharmacy, Mahidol University and MS-222 was purchased from Sigma Inc. (St-Louis, MO, USA).

24-hr LC_{50} of synthetic eugenol, clove oil and MS-222

The experiment consisted of two components, the range finding test and the definitive test. The range finding test was used to determine the concentration range of the anesthetic, by identifying the lowest level that caused 100% mortality and the highest level that caused 0% mortality in 24 h, which was then used in the definitive test. After the concentrations from the range finding test were obtained, each concentration was divided into a logarithmic-spaced series for the final concentrations in the definitive test. The experiment was conducted in glass aquaria, which contained 2.5 L of water with continuous aeration. The mean water temperature throughout the 24-hr test was 26.7±1.67 °C and the water dissolved oxygen levels were greater than 85% saturation. Anesthetic stock solution was added to produce final concentrations of 0, 5, 10, 15, 20, 25 and 30 ppm for synthetic eugenol and

clove oil while concentrations of MS-222 were 30, 45, 60, 75, 90 and 105 ppm. Ten fish were randomly selected from an acclimated tank and placed in each glass aquaria. Total mortality, behavior, temperature and dissolved oxygen were measured every 3 hr for the first 12 hr of the experiment and every 6 hr for the remaining 12 hr. Fish were considered dead when no opercular movement was observed for 15 min continuously. The experiment was conducted in three replicates. The 24-hr LC_{50} value of each anesthetic was analyzed using probit analysis as described by Finney (1971).

Onset and recovery from anesthesia

The high efficacy of an anesthetic is determined by its ability to make fish easy to handle with an induction time of 3 min or less, to allow the fish to recover in 10 min or less and to cause no mortality after a 15 min exposure (Marking and Meyer, 1985). In this study, evaluation of the stages of anesthesia and recovery were developed from criteria outlined by Summerfelt and Smith (1990) and Iwama and Ackerman (1994), respectively. The experiment was performed in glass aquaria, which were filled with 2.5 L of dechlorinated tap water. Water quality was controlled as for the 24-hr LC_{50} test. The final concentrations of eugenol and clove oil used were 0, 5, 10, 15, 20 and 25 ppm, while the concentrations of MS-222 used were 30, 45, 60, 75, 90, 105 and 120 ppm. Each concentration of each anesthetic was added directly to the water and the water was vigorously aerated for 5 min prior to each experiment. Ten fish were transferred individually to each treatment aquarium containing each concentration of anesthetic. Three replications were used. Tested fish were exposed to the anesthetic for 20 min. The time to achieve each stage of anesthesia onset and the behavior of exposed fish were recorded. After the 20 min, tested fish were immediately transferred to a 20-L recovery tank with continuous aeration; recovery time was recorded. Fish were placed in the recovery tank for seven days and fed with pellet feed in order to observe abnormal behavior and mortality.

Statistical analysis

One-way analysis of variance was applied to check for significant differences between the average values of the tested parameters in the different groups. Duncan's new multiple range test (DMRT) was used to determine the difference between groups. Differences were considered statistically significant at a *p-value = 0.05*.

RESULTS

24-hr LC_{50} of synthetic eugenol, clove oil and MS-222

The static acute toxicity test with tilapia fry indicated that 24-hr LC_{50} of synthetic eugenol, clove oil-derived eugenol and MS-222 was 16.98, 16.95 and 72.50 ppm, respectively (Table 1). The safety margin of synthetic and clove oil-derived eugenol was 0.169 ppm, while for MS-222 it was 0.725 ppm. Upper and lower limits at 95% are shown in Table 1. Total mortality of tilapia fry

Table 1 24-hr LC_{50} of synthetic eugenol, clove oil-derived eugenol and MS-222 in fry of Nile tilapia (*Oreochromis niloticus* Linn.).

Type of anesthetic	LC_{50} (ppm)	Lower limit at 95% (ppm)	Upper limit at 95% (ppm)	Safety of margin (ppm)
Synthetic eugenol	16.98	16.35	17.60	0.169
Clove oil-derived eugenol	16.95	16.25	17.65	0.169
MS-222	72.50	71.61	73.39	0.725

occurred when they were exposed to 25 ppm of clove oil within 6 hr, while all fish exposed to 90 ppm MS-222 died within 18 hr.

Onset and recovery from anesthesia

Onset and recovery from anesthesia were measured by the induction time and recovery time (mean±SE) and these data are shown in Tables 2-4. The significant differences in the induction times and recovery times depended on the concentration of the anesthetic solution.

In Table 2, a synthetic eugenol dose of 5 ppm induced tilapia fry to achieve the sedation stage of anesthesia (stage 1 of anesthesia). Fry reached stage 5 of anesthesia (loss of reflex reactivity) in 3.40±0.14 min when exposed to 20 ppm of eugenol. The recovery time from stage 5 was 7.70±0.11 min (mean±SE). All tilapia fry were induced to every stage of anesthesia when exposed to various induction doses, except stage 4 for the exposure to 10 ppm of synthetic eugenol because there was only 76.66% induced fry at this stage. There was no mortality at any dosage of synthetic eugenol during the 20-min exposure.

There was a similar result in the efficacy of synthetic eugenol (Table 2) and clove oil-derived eugenol (Table 3). Tilapia fry were calmed in the sedation stage when exposed to 5 ppm of clove oil-derived eugenol and reached stage 5 of anesthesia within 3 min when exposed to 20 ppm. Recovery time from stage 5 of anesthesia was 5.26±0.11 min (mean±SE). Every stage of anesthesia was achieved with the various doses with 100% induction. There was 6.66% mortality of tilapia fry exposed to 25 ppm clove oil during 20 min, in which stage 6 was achieved.

Table 4 shows the results of the efficacy of MS-222 as an anesthetic. The sedation stage of anesthesia in tilapia fry was induced after exposure to 30 ppm of MS-222. Stage 5 of anesthesia was achieved within 2.16±0.02 min when exposed to 120 ppm of MS-222 and it required 12.6±0.34 min of recovery time after exposure at this

Table 2 Mean (±SE) (n=30) induction and recovery times for tilapia fry exposed to various concentrations of synthetic eugenol for a period of 20 min.

Conc. (mg/L)	Induction time (min)						Recovery time (min)	Mortality (%)
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6		
5	13.89±0.25 ^{a}	na	na	na	na	na	0.35±0.01 ^{a}	0
10	1.43±0.03 ^{b}	3.11±0.05 ^{a}	6.04±0.14 ^{a}	13.23±0.40 ^{a}	na	na	3.37±0.14 ^{b}	0
15	1.21±0.05 ^{b}	2.00±0.05 ^{b}	2.86±0.07 ^{b}	4.20±0.14 ^{b}	6.99±0.27 ^{a}	na	6.09±0.13 ^{c}	0
20	0.35±0.02 ^{c}	0.84±0.05 ^{c}	1.35±0.02 ^{c}	2.09±0.05 ^{c}	3.40±0.14 ^{b}	na	7.70±0.11 ^{d}	0
25	0.23±0.01 ^{c}	0.44±0.01 ^{d}	1.08±0.02 ^{d}	1.36±0.02 ^{d}	2.14±0.06 ^{c}	na	10.77±0.23 ^{e}	0

Note: na = that stage of anesthesia was not achieved after 20 min of exposure.
Means with different superscripts within the same column were significantly different at *p value* = 0.05.

Table 3 Mean (±SE) (n=30) induction and recovery times for tilapia fry exposed to various concentrations of clove oil-derived eugenol for a period of 20 min.

Conc. (mg/L)	Induction time (min)						Recovery time (min)	Mortality (%)
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6		
5	13.40±0.47 [a]	Na	na	na	na	na	0.33±0.05 [a]	0
10	2.69±0.09 [b]	4.10±0.09 [a]	8.26±0.24 [a]	15.85±0.35 [a]	18.10±0.65 [a]	na	2.45±0.04 [b]	0
15	0.83±0.06 [c]	1.58±0.06 [b]	3.02±0.09 [b]	5.31±0.36 [b]	7.61±0.32 [b]	na	5.14±0.18 [c]	0
20	0.34±0.05 [c]	0.75±0.11 [c]	1.36±0.06 [c]	1.98±0.09 [c]	2.86±0.16 [c]	na	5.26±0.11 [c]	0
25	0.28±0.02 [c]	0.58±0.06 [c]	0.85±0.07 [d]	1.18±0.08 [d]	2.01±0.14 [c]	19.40±0.13	7.44±0.29 [d]	6.66

Table 4 Mean (±SE) (n=30) induction and recovery times for tilapia fry exposed to various concentrations of MS-222 for a period of 20 min.

Conc. (mg/L)	Induction time (min)						Recovery time (min)	Mortality (%)
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6		
30	15.82±0.31 [a]	na	na	na	na	na	0.29±0.02 [a]	0
45	7.93±0.30 [b]	16.87±0.57 [a]	na	na	na	na	1.11±0.05 [a]	0
60	1.75±0.08 [c]	4.43±0.22 [b]	6.77±0.22 [a]	18.27±0.64 [a]	na	na	2.98±0.10 [ab]	0
75	1.18±0.04 [d]	2.21±0.05 [c]	3.52±0.06 [b]	13.01±0.35 [b]	na	na	2.72±0.10 [ab]	0
90	0.48±0.01 [e]	1.26±0.01 [d]	2.74±0.06 [c]	6.37±0.18 [c]	13.03±2.04 [a]	na	4.00±0.07 [b]	0
105	0.30±0.01 [e]	0.60±0.04 [e]	1.2±0.04 [d]	2.15±0.06 [d]	4.07±0.19 [b]	19.25±0.21 [a]	9.15±2.77 [c]	26.66
120	0.22±0.01 [e]	0.35±0.01 [e]	0.55±0.17 [e]	1.44±0.01 [d]	2.16±0.02 [c]	9.08±0.24 [b]	12.6±0.34 [d]	50

concentration for 20 min. In this study, 60% of fish were exposed to 45 ppm MS-222 to achieve stage 2 of anesthesia, while there was only 26.66% of fish exposed to 60 and 105 ppm MS-222 to achieve stage 4 and stage 6 of anesthesia, respectively. However, there was 26.66% mortality at 105 ppm and 50% mortality at 120 ppm of MS-222 during 20-min exposure.

The comparison of induction time to sedation stage (stage 1), stage 5 of anesthesia and recovery from anesthesia are shown in Figure 1. Synthetic and clove oil-derived eugenol had a lower induction dose than MS-222. With all types of anesthetic, the recovery time of fish exposed to a high dose was longer than for a low dose.

DISCUSSION

Eugenol (2-methoxy-4-(2-propenyl) phenol) has been investigated for its efficacy as an anesthetic for many species of fish, such as silver perch (*Bidyanus bidyanus*) (Kildea *et al*., 2004), rabbitfish (*Siganus lineatus*) (Soto and Burhanuddin, 1995), fathead minnows (*Pimephales promelas* Rafinesque, 1820) (Palic *et al*., 2006) and prawns (*Macrobrachium rosenbergii*) (Coyle *et al*., 2005; Saydmohammed and Pal, 2009). This anesthetic has various benefits, such as no withdrawal time is required, it is non-carcinogenic and non-mutagenic, it can be used at low concentration and it is inexpensive. Moreover, this anesthetic can be used as a tranquilizer, narcoanesthetic and muscle relaxant or paralytic drug in humans (Guenette *et al*., 2007). The analgesic effects of eugenol result from the inhibition of prostaglandin H synthase (PHS). The major area of entry and excretion of anesthetic in fish is through the gills and the rate of passage through the gills depends mainly on its degree of ionization and lipid solubility (Keene *et al*., 1998).

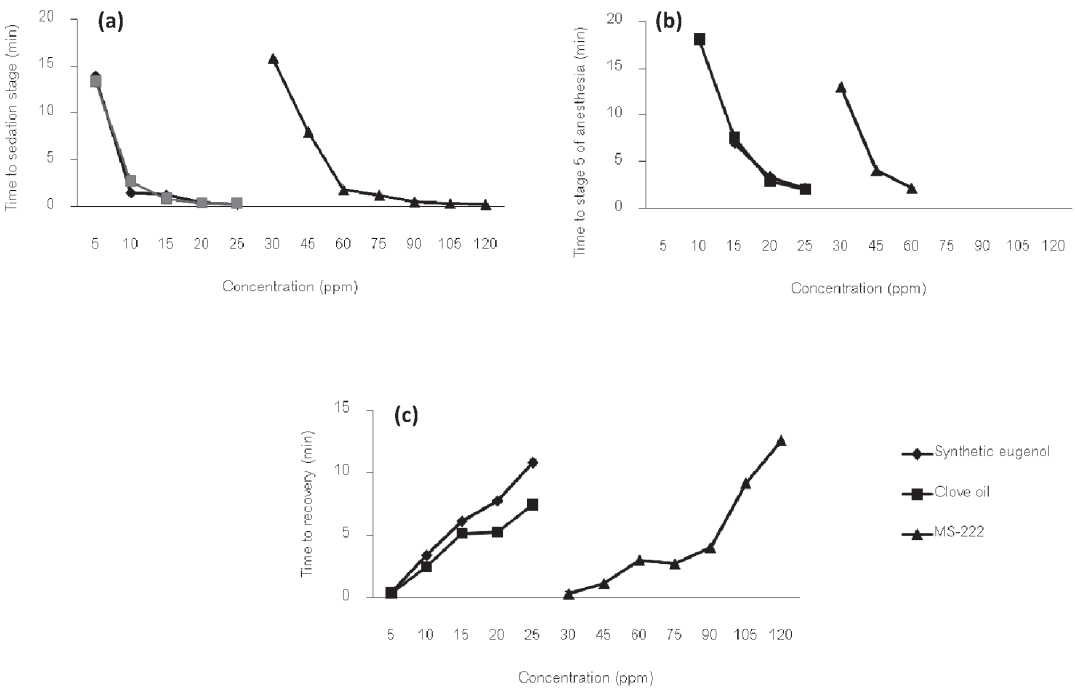

Figure 1 Time required to achieve (a) sedation stage (stage 1); (b) stage 5 of anesthesia; and (c) and recovery in tilapia fry with various concentrations of synthetic eugenol, clove oil and MS-222. Data points represent the mean (n=30).

Previously, clove oil-derived eugenol had been extensively studied in fish, but there were various steps to obtain the anesthetic. Therefore, synthetically derived eugenol (100% eugenol by weight) was developed because it is as effective as clove oil-derived eugenol and it is easy to prepare.

This study showed clearly that synthetic eugenol was an effective anesthetic in tilapia fry. Tilapia fry exposed to synthetic eugenol progressed sequentially through the various stages of anesthesia outlined by Summerfelt and Smith (1990).

The 24-hr LC_{50} of synthetic eugenol for tilapia fry was 16.98 ppm. When compared with clove oil-derived eugenol and MS-222, synthetic eugenol had a similar value to clove oil-derived eugenol (16.95 pmm), but this was much lower than for MS-222 (72.50 ppm). When a safety margin is taken into account, the results suggested that synthetic and clove oil-derived eugenol could be used safely at low doses. The acute toxicity of eugenol and clove oil in fish was different depending on the species and size of fish, exposure time and concentration of active ingredient. Some fish species that have been studied using an acute toxicity test with eugenol and clove oil include rainbow trout (*Oncorhynchus mykiss*, Walbuam), sea bass (*Lates calcarifer*, Bloch) and zebra fish (*Danio rerio*, Hamilton) (Keene *et al*., 1998; Hangono, 2003; Grush *et al*., 2004).

Synthetic eugenol and clove oil-derived eugenol could induce tilapia to various stages of anesthesia with a lower dose and more quickly than MS-222; they met and exceeded the first criterion of Marking and Meyer (1985). Both synthetic and clove oil-derived eugenol had a greater safety margin than MS-222 because they produced effects at a low dose similar to those of a higher dose, while MS-222 did not. Similar results were reported by Keene *et al*. (1998) and Grush *et al*. (2004). In this study, fish achieved sedation when exposed to 5 ppm of synthetic and clove oil-derived eugenol. Cooke *et al*. (2004) also recommended this dose to sedate largemouth bass (*Micropterus salmoildes*) for transport. Moreover, Cho and Heath (2000) had recommended 20 ppm clove oil-derived eugenol to achieve stage 5 of anesthesia. This recommended dose was similar to the effective dose in this study. The results from this study also indicated that eugenol from both sources was more effective than MS-222 when induction of sedation and stage 5 were considered. It took 30 ppm of MS-222 to achieve sedation and 90 ppm to achieve 100% of stage 5 without mortality, but the induction time of 13.03 ± 2.04 min was much longer than the required criterion of 3 min.

In terms of recovery, a high dose of all anesthetics in the current study caused a longer recovery period than for a low dose. Considering the dose required to induce stage 5 of anesthesia, the recovery time of synthetic eugenol was longer than for clove oil-derived eugenol. The previous study by Guenette *et al*. (2007), which investigated the pharmacokinetics of eugenol in rainbow trout (*Oncorhynchus mykiss*), suggested that eugenol had a half life of 12.14 h and it was well absorbed and eliminated by exposed fish. Eugenol had greater effects on the respiratory and cardiac system than MS-222 resulting in a slower heart and respiratory rate, which caused a longer retention of eugenol in the blood stream (McFarland, 1959; Keene *et al*., 1998). This reason explained why eugenol elicited stages of anesthesia sooner, had a longer recovery time and required a lower dose than MS-222.

The current study suggested that synthetic eugenol had higher efficacy as an anesthetic than MS-222, but it was similar to clove oil-derived eugenol. Thus, synthetic eugenol should be used as the alternative anesthetic in many activities related to fish handling in aquaculture.

CONCLUSION

Synthetic eugenol can be used as an anesthetic for tilapia and other aquatic animals because it satisfies the eight criteria of an ideal anesthetic (Marking and Meyer, 1985). Its main advantages are low cost, no withdrawal time requirements and its relative safety to fish, users and the environment. This study indicated that synthetic eugenol has the same efficacy as clove oil-derived eugenol, but was more effective than MS-222. The recommended dose of synthetic eugenol to achieve stage 5 of anesthesia in tilapia fry was 20 ppm, which could induce rapid anesthesia (about 3 min) with a relatively short time for recovery. For transportation periods up to 6-8 hr, 5 ppm of synthetic eugenol may be considered to reduce metabolism during transport.

ACKNOWLEDGEMENTS

This research was supported by Kasetsart University Research and Development Institute and The Thailand Research Fund.

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