

## Anesthetic activity of plant essential oils on *Cyprinus carpio* (koi carp)

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### Summary

The aims of this study were to investigate the anesthetic and cytotoxic effects of essential oils (EOs) of *Ocimum basilicum* (OBO), *O. canum* (OCO), and *O. sanctum* (OSO) on *Cyprinus carpio* (koi carp). For anesthetic effect, induction time to surgical anesthesia and recovery time were determined. For cytotoxicity effect, viability of fish peripheral blood nuclear cells (PBMCs) was investigated. Results indicated that increasing oil concentration caused significant ( $p < 0.01$ ) decrease of induction time. OSO at 100, 200, and 300 mg/L gave the induction time of  $169.5 \pm 10.2$ ,  $62.8 \pm 2.3$ ,  $45.3 \pm 2.2$  sec, respectively, significantly shorter than OCO, and OBO. The recovery time of anesthetized fish was dose dependent ( $p < 0.01$ ). Among them, OCO showed the longest recovery time of  $313.0 \pm 8.1$ ,  $420.7 \pm 12.6$ ,  $616.6 \pm 12.1$  sec for concentrations of 100, 200, and 300 mg/L, respectively, followed by OSO and OBO, respectively. Within 10 min contact time of the EOs and fish PBMCs, the fish PBMC viability was higher than 80%. Increase contact time and EO concentration caused an increase in cytotoxicity to fish PBMC. OBO showed less toxic than OSO and OCO. Based on the desired induction and recovery times for anesthetizing koi carp, OBO, OCO, and OSO at 300, 200, and 100 mg/L, respectively were suggested to be the most suitable. It was concluded that OBO, OCO, and OSO can be used as natural anesthetics for fish.

**Keywords:** Anesthetic activity, cytotoxicity, *Ocimum basilicum*, *Ocimum canum*, *Ocimum sanctum*

### 1. Introduction

Stress in fish can be induced by physical, chemical, and perceived factors. These factors can evoke several physiological responses in fish (1). Handling, capture, transport, and confinement are the most common physical stress factors. Generally, when fish are subjected to the physical stress they show physiological stress response by escape attempts (2). Physical stress can

cause many undesirable responses such as the changes in circulating catecholamines and corticosteroids, resulted in a changes in metabolism, haematological features, immune function features, stress protein, osmoregulatory disturbance, reduction of egg quality, spermatocrit, and growth as well as a decrease in disease resistance leading to increase of susceptibility to diseases or illness (3,4).

Normally, anesthetics have been used to immobilize and reduce metabolism in fish, thereby improve stress response of fish (5). There are several chemical anesthetics available for aquatic animals, such as tricaine methanesulfonate (MS-222), benzocaine, quinaldine, 2-phenoxyethanol, and metomidate (6). These anesthetics involve the central nervous system by either enhancing inhibitory signal or blocking excitatory signals with different target binding (7). The choice of anesthetics depends on several factors such as safety,

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cost, and comfortable use (8). However, these chemical anesthetics have a long withdrawal period before human consumption (9). MS-222 is currently approved by the United States Food and Drug Administration (10), but the fish exposed to MS-222 require a long withdrawal period (21 days) for being released into the environment or sold for human consumption (11). Importantly, MS-222 is suspected as a carcinogenic agent in human (12). According to these issues, anesthetics from natural sources such as plants, particularly the edible plants, are of increasing interest.

The essential oil (EO) of certain plants has been recognized to have anesthetic activity. Clove oil, an EO from flower buds of clove tree (*Syzygium aromaticum*) is the most common agent used to immobilize fish (13,14). The anesthetic activity of clove oil is considered to be due to eugenol and eugenol derivatives existing in the EO (15). Literature review on phytochemistry and the related activities demonstrates that eugenol containing plant has anesthetic effect in fish. Moreover, EO from *Ocimum gratissimum* which is rich in eugenol, has been reported to have anesthetic activity in *Rhamdia quelen* (silver catfish) (16) and *Paralichthys orbignyanus* (Brazilian flounder) (17). Interestingly, the EOs of certain plants in Lamiaceae such as *O. basilicum*, *O. canum*, and *O. sanctum* were reported to contain eugenol and its derivatives (18-20). However, they have not yet been studied or used for fish anesthesia. Plants in genus *Ocimum* are found primarily in the tropical regions of Asia, Africa, and central and south America (21). *O. basilicum*, *O. canum*, and *O. sanctum* are edible plants and have been historical used as food or food additives in Thai and other Asian cuisines. The use in food remedies of these plants can basically prove their safety for human consumption. They also have been used as Asian folk medicine because they have been reported to exert a variety of biological activities such as anti-proliferative activity against cancer cells, anti-microbial and anti-fungal activities (22,23), and anti-oxidative activity (24). These plants are widely grown in Thailand and many Asian countries. Their advantages on low cost, safety, ease of raw plant material collecting, and high existing of potential compounds (eugenol and eugenol derivatives) are of most interest for investigation as an alternative fish anesthetics.

Koi carp, the Japanese ornamental carp, became an appreciated and expensive pet worldwide (25). Due to their distinctive color and scale patterns, this fish becomes the most popular for outdoor ornamental (26). Currently, handling and transportation of this fish is increasing leading to the increase in demand for anesthetics to decrease the fish stress according to these physical processes. Therefore, koi carp was selected to use as a fish model in the present study. This study aimed to investigate the anesthetic efficacy of EO from Thai medicinal plants (*O. basilicum*, *O. canum*, and *O. sanctum*) on this fish and cytotoxic effect of these EOs

was also evaluated for safety issue.

## 2. Materials and Methods

### 2.1. Chemicals

Absolute ethanol and phosphate buffer solution (PBS) were from Merck Millipore (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and MS-222 were of Sigma-Aldrich (St Louis, MO, USA). Lymphoprep was from Axis-Shield PoC AS (Oslo, Norway). RPMI 1640, fetal bovine serum, L-glutamine, and penicillin-streptomycin were from GIBCO Invitrogen™ (New York, USA).

### 2.2. Fish and rearing conditions

Healthy juvenile (weight;  $9.7 \pm 0.1$  g and total length;  $9.2 \pm 0.1$  cm) and adult (weight;  $251.4 \pm 4.6$  g and total length;  $20.9 \pm 2.7$  cm) koi carp were purchased from an ornamental fish shop located in Chiang Mai, Thailand. The fish were stocked in a 300 L tank and maintained under laboratory conditions. During acclimatization, the dechlorinated tap water was changed daily (50%). The fish were fed with a commercial pelletized diet (INTEQC Feed, Thailand) and held under natural light condition. After maintenance at these conditions for 2-4 weeks, the fish were starved for 24 h prior to the experiments. The studies were conducted according to a permission obtained by the Animal Care and Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC) (Process no. R3/2555).

### 2.3. Plant materials

*O. basilicum*, *O. canum*, and *O. sanctum* were collected from the medicinal plant garden of Chiang Mai University, Chiang Mai, Thailand in December 2015. After identification, the voucher specimens were deposited at the Herbarium, Faculty of Pharmacy, Chiang Mai University, Thailand (collection No. 009802, 009819, and 009810, respectively).

### 2.4. Extraction and analysis of EOs

OBO, OCO, and OSO were obtained from the fresh aerial parts of *O. basilicum*, *O. canum*, *O. sanctum*, respectively by hydro-distillation for 3 h. The obtained EOs of each plant was stored in a light-resistant container at  $-20^{\circ}\text{C}$  for further studies.

Chemical analysis of the obtained EOs was performed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 gas chromatography coupled to electron impact (70 eV) and a Hewlett Packard (HP) mass selective detector (HP 5973-MSD). The HP5-MSI; 30.0 m  $\times$  0.25 mm

**Table 1. Behavior of *Cyprinus carpio* (koi carp) in various stages of anesthesia and recovery from anesthesia**

Stages	Description	Exhibited behavior
0	Normal	Normal
1	Light sedation	Fish are disoriented
2	Excitatory stage	Fish reduced swimming activity and show partial loss of equilibrium
3	Surgical stage	Fish stopped their swimming activity, experienced a total loss of equilibrium, and had no responsiveness
4	Death stag	Death; respiration stopped
Recovery		Retaking of swimming activity, equilibrium, and responsiveness

i.d.  $\times$  0.25  $\mu$ m film thickness (Agilent Technologies Inc, USA) was used as a capillary column for GC-MS. The analytical conditions used were modified from the previous study (27). Briefly, the injector temperature was 250°C, the oven temperature conditions were as follows: 3 min isothermal at 70°C, then increased at the rate of 3°C/min to 188°C and then at 20°C/min to 280°C (3 min isothermal), and the detector temperature was 280°C. The EO sample was diluted with dichloromethane to 1:100 (v/v). Injection volume was 1  $\mu$ L. Identification of the compounds was performed based on comparing their retention times and mass spectra relative to those of spectral peaks available in Wiley and the National Institute of Standards and Technology; NIST mass spectral libraries.

### 2.5. *In vivo* anesthetic activity of EOs

The anesthetic activity of OBO, OCO, and OSO was studied in juvenile koi carp. MS-222 was used as a positive control. The EO was diluted to 1:10 (v/v) with absolute ethanol. MS-222 was dissolved in deionized water to a concentration of 10 g/L and adjusted to pH 6.5-7.5 by sodium bicarbonate. Two hundred sixty fish were divided into 13 groups, each group contained 20 individual fish in an aquarium containing 7 L of dechlorinated tap water (15  $\times$  30  $\times$  22.5 cm) and used for each EO type and concentration. The tanks (10  $\times$  10  $\times$  15 cm glass aquarium) contained 1 L of dechlorinated water were prepared for anesthesia tanks. The diluted EO or MS-222 stock solution was added to the tank until reaching the final concentrations of 100, 200, and 300 mg/L. A tank contained 0.3% (v/v) absolute ethanol in dechlorinated water was used as a vehicle control. The ethanol concentration of the control tank was the same as the maximum amount used in the anesthetic tank. The fish was placed into the anesthesia tank one by one and each fish was used only once. The anesthetic activity was evaluated by comparing the induction time that the fish required to reach a surgical stage of anesthesia after exposure to the EO. The stages of anesthesia were assessed in accordance with fish behavioural responses adapted from McFarland (28) and Zahl *et al.* (29) (as described in Table 1). The body part near the caudal fin of the fish was pressed with forceps in order to confirm that the fish reached stage 3

which is a surgical stage of anesthesia. The maximum observation time was 20 min. After reaching at the surgical stage of anesthesia, the fish were immediately transferred to anesthetic free tank to be recovered from the anesthesia. The recovery time was recorded after the fish completely recovered.

### 2.6. Cytotoxicity study of EOs

Determination of normal peripheral blood nuclear cells (PBMCs) was performed using MTT colorimetric assay. This assay is based on the ability of viable cells to metabolize a water-soluble tetrazolium salt into a water-insoluble formazan product. Thirty adult koi carp were placed one by one into the anesthesia tank (20  $\times$  40  $\times$  25 cm) containing 100 mg/L MS-222 in 15 L of dechlorinated tap water in order to be anesthetized to stage 3 of anesthesia. Blood samples (20 mL) from six healthy fish were collected by venipuncture of the caudal vessel and kept in heparin-coated test tubes. The fish blood was diluted (1:1 v/v) with 0.1 M phosphate buffer solution (PBS) and further diluted (3:1 v/v) with Ficoll-Hypaque. The fish PBMCs were collected after centrifuging at 3,000 rpm for 30 min and washed three times with PBS. Complete RPMI 1640 medium (10% FBS, 100 unit/mL of penicillin, 100  $\mu$ g/mL of streptomycin, and 1 mM of L-glutamine) was used to resuspend PBMCs. The PBMCs (1  $\times$  10<sup>6</sup> cells) suspension in 100  $\mu$ L medium were seeded into 96-well plates and incubated for 24 h at 37°C, 5% CO<sub>2</sub> and 95% relative humidity. An exact amount of 100  $\mu$ L of the fresh medium containing various concentrations (100, 200, 300, 400, and 500 mg/L) of EOs dissolved in DMSO (100 mg/mL) was added into each well and incubated for 10 min, 1, 6, and 12 h. After that, 100  $\mu$ L of the medium was removed, 15  $\mu$ L of MTT dye solution (5 mg/L in PBS) was added to each well. After 4 h of incubation, the supernatant was removed and 200  $\mu$ L DMSO was added to each well to dissolve formazan crystals. Using an AccuReaderTM M965/965+ microplate reader (Metertech Inc., Taiwan), the absorbance was measured at 578 nm and corrected by a reference wavelength of 630 nm. Solution of 1.0% of DMSO in RPMI 1640 complete medium was used as a reference control. All experiments were performed in triplicate. The percent of cell viability was calculated

using the following equation.

$$\text{Cell viability (\%)} = \frac{\text{mean absorbance of treatment}}{\text{mean absorbance of reference}} \times 100$$

### 2.7. Statistical analysis

Data are presented as means  $\pm$  S.E.M. and normality of the data was checked using Kolmogorov-Smirnov's test. The ANOVA followed by Tukey's post hoc test was used to analyze anesthetic activity and cytotoxicity study. The values of  $p < 0.05$  were considered as statistically significant.

## 3. Results

### 3.1. Extraction and analysis of EOs

Hydrodistillation for 3 h of three plants yielded different amount of EOs. The highest yield (0.24%) was from *O. basilicum*. The yields of EOs obtained from *O. canum* and *O. sanctum* were only 0.14% and 0.18%, respectively. OBO appeared as a clear pale

yellowish liquid similar to OSO. The outer appearance of OCO was slightly different. It was a clear liquid with intense yellowish orange. GC-MS analysis of OBO showed that there were 20 identifiable components which represented 96.74% of the total compounds in the EO whereas there were 13 compounds presented in OCO and 14 compounds in OSO, which represented 92.09% and 91.27% of the total compounds in OCO and OSO, respectively (Table 2). The major component of OBO was methyl chavicol (78.12%). Other main compounds existing in this EO but far less amount than methyl chavicol were 1,8-cineole (3.54%) and trans- $\alpha$ -bergamotene (3.02%). Eugenol was not found in OBO but methyl eugenol was found instead but at very low amount (0.44%). Major compounds of OCO were E-citral (41.01%) and Z-citral (37.04%) whereas that of OSO were methyl eugenol (46.18%) and eugenol (11.56%).

### 3.2. Anesthetic activity of EOs

It was found that increasing concentration of EO as well as MS-222 significantly ( $p < 0.05$ ) decreased the induction time for fish anesthesia (Figure 1). No

**Table 2. Chemical compounds existing in OBO, OCO, and OSO**

Retention time (min)	Components	Percentage of chemical compound		
		OBO	OCO	OSO
4.08	$\alpha$ -Pinene	0.09	0.09	0.09
4.39	Camphene	-	-	-
4.93	Sabinene	0.09	0.09	0.09
5.03	$\beta$ -Pinene	0.17	0.17	0.17
5.33	$\beta$ -Myrcene	0.29	0.29	0.29
6.43	Limonene	0.19	0.19	0.19
6.60	1,8-Cineole	3.54	3.54	3.54
7.02	1,3,6-Octatriene	2.37	2.37	2.37
8.35	Terpinolene	0.16	0.16	0.16
8.79	Linalool	0.53	0.53	0.53
10.42	L-Camphor	1.49	1.49	1.49
11.23	Borneol	0.57	0.57	0.57
11.32	$\alpha$ Terpineol	-	-	-
13.04	Methyl chavicol	78.12	78.12	78.12
14.74	Z-Citral (Neral)	-	-	-
15.38	Geraniol	-	-	-
16.11	E-Citral (Geranial)	-	-	-
18.66	$\alpha$ -Cubebene	-	-	-
19.23	Eugenol	-	-	-
19.76	$\alpha$ -Copaene	-	-	-
20.55	$\beta$ -Elemene	0.70	0.70	0.70
21.43	Methyl eugenol	0.44	0.44	0.44
21.81	$\beta$ -Caryophyllene	-	-	-
22.21	Trans- $\alpha$ -Bergamotene	3.02	3.02	3.02
22.79	$\beta$ -Selinene	0.34	0.34	0.34
23.08	$\beta$ -Farnesene	-	-	-
23.90	Germacrene-D	1.36	1.36	1.36
24.10	Trans- $\beta$ -Farnesene	0.23	0.23	0.23
25.10	$\beta$ -Bisaboloene	-	-	-
25.66	$\beta$ -Sesquiphellandrene	0.25	0.25	0.25
26.48	Cis- $\alpha$ -Bisabolene	-	-	-
30.08	$\delta$ -Cadinene	2.79	2.79	2.79
Total		96.74	96.74	96.74

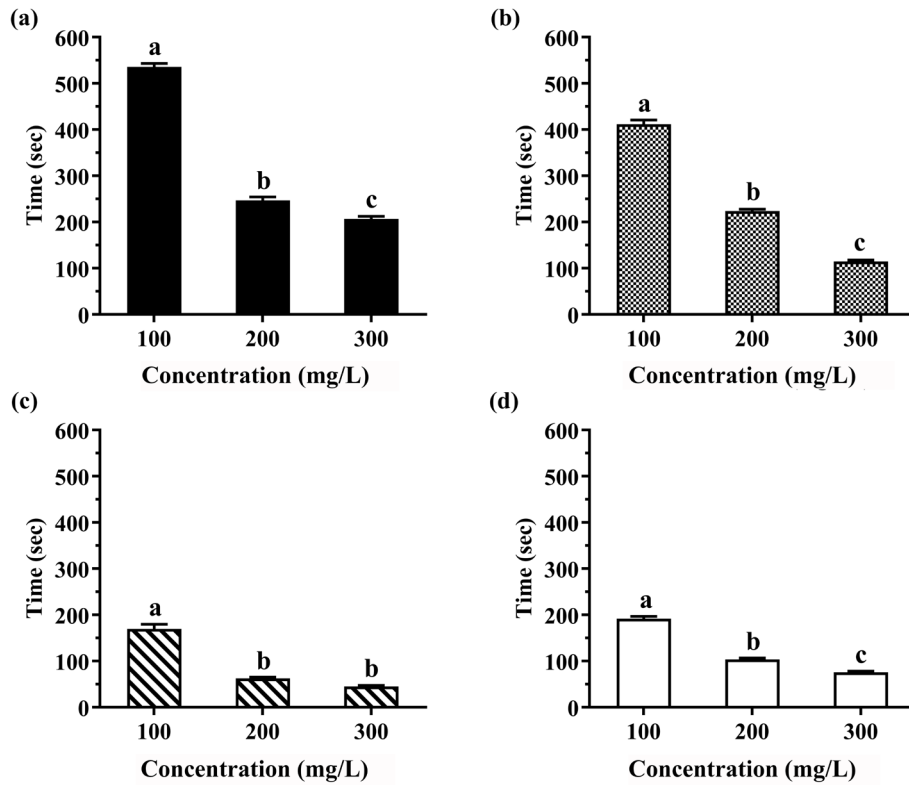


Figure 1. Induction time for koi carp ( $n = 20$ ) exposed to 100, 200, and 300 mg/L of OBO (a), OCO (b), OSO (c), and MS-222 (d) to reach the surgical anesthesia stage. Data are presented as means  $\pm$  S.E.M. ( $p < 0.001$ ).

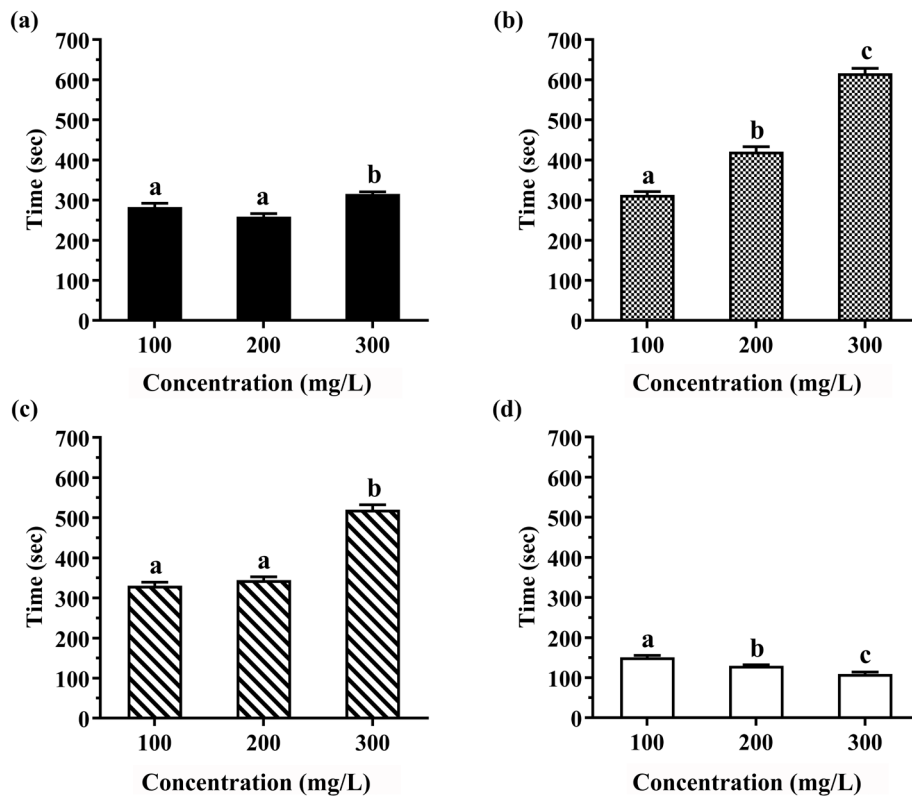


Figure 2. Recovery time for koi carp ( $n = 20$ ) exposed to 100, 200, and 300 mg/L of OBO (a), OCO (b), OSO (c), and MS-222 (d). Data are presented as means  $\pm$  S.E.M. ( $p < 0.01$ ).

mortality of the fish was found after receiving the EOs at all tested concentrations (100, 200, and 300 mg/L). Data analysis using two-way ANOVA indicated that not only EO type but also EO concentration played a significant role on anesthetic effect ( $p < 0.001$ ). Moreover, the EO type  $\times$  EO concentration interaction also showed significant ( $p < 0.001$ ). Within the same final concentration, OSO revealed the shortest induction time of all stages of anesthesia. The induction times for anesthesia (stage 3) caused by OSO at 100, 200, and 300 mg/L were  $169.5 \pm 10.2$ ,  $62.8 \pm 2.3$ ,  $45.3 \pm 2.2$  sec, respectively. Moreover, it was found that at these concentrations, the induction time to the surgical anesthesia of the fish caused by OSO was significantly shorter than that caused by MS-222 ( $p < 0.05$ ). OBO at 100, 200, and 300 mg/L showed longest induction times of approximately 535, 250, and 205 sec, respectively. It was observed that after exposure to OBO and OCO, the fish showed slightly mucous secretions.

The EO type and concentration also showed a major effect on the recovery time ( $p < 0.001$ ) (Figure 2). The EO type  $\times$  concentration interaction showed significant ( $p < 0.001$ ). While ethanol at 0.3% v/v neither sedated the fish nor provided any undesirable side effect to the fish. The recovery time of the fish anesthetized by MS-222 was faster than those anesthetized by the EOs and the effect was not dose dependent. The recovery time of the fish anesthetized by the EOs was dose dependent ( $p < 0.01$ ). The anesthetized fish caused by OCO showed the longest recovery time at concentration of 100, 200, and 300 mg/L ( $313.0 \pm 8.1$ ,  $420.7 \pm 12.6$ ,  $616.6 \pm 12.1$  sec, respectively), followed by those anesthetized

by OSO ( $330.4 \pm 8.5$ ,  $344.5 \pm 8.1$ ,  $520.0 \pm 12.3$  sec, respectively) and OBO ( $282.9 \pm 9.0$ ,  $258.6 \pm 7.7$ ,  $315.1 \pm 5.4$  sec, respectively).

### 3.3. Cytotoxicity study of EOs

Direct contact of fish PBMCs to the EOs demonstrated some difference. All tested EOs exhibited concentration and time dependent toxicity to the fish but at different levels ( $p < 0.05$ ) (Figures 3-5). OBO showed the least toxicity among them. More than 80% of fish PBMCs survived after exposure to all concentrations of the EOs (100, 200, 300, 400, and 500 mg/L) for 10 min and those exposed to 100 mg/L OBO for 1, 6, and 12 h. Although an elevation of EO concentration and exposure time increased toxicity to the fish PBMCs, at high concentrations of 300-500 mg/L, OBO showed significantly lower cytotoxic than OCO and OSO.

## 4. Discussion

Our investigation started with the extraction of EOs from the fresh raw plant materials followed by chemical analysis of the EOs using GC-MS and then the *in vivo* test in koi carp. We found that OBO, OCO, and OSO had strong effect on fish anesthesia. The yield of OBO obtained was similar with that extracted from *O. basilicum* grown in Australia (30), whereas the yield of OCO and OSO from *O. canum* and *O. sanctum*, respectively, less than the previous reports (31,32). The variation of yield as well as chemical races in the producing plant species are often affected by

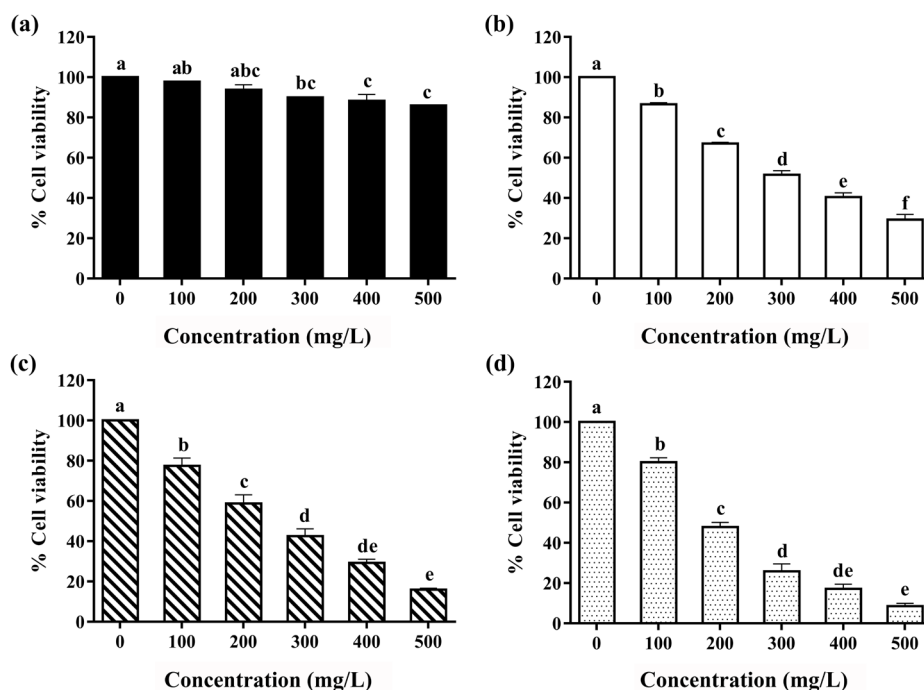


Figure 3. Dose-response curves of viability of koi carp PBMCs exposed to OBO at 10 min (a), 1 h (b), 6 h (c), and 12 h (d). Data are presented as means  $\pm$  S.E.M. ( $p < 0.05$ ).

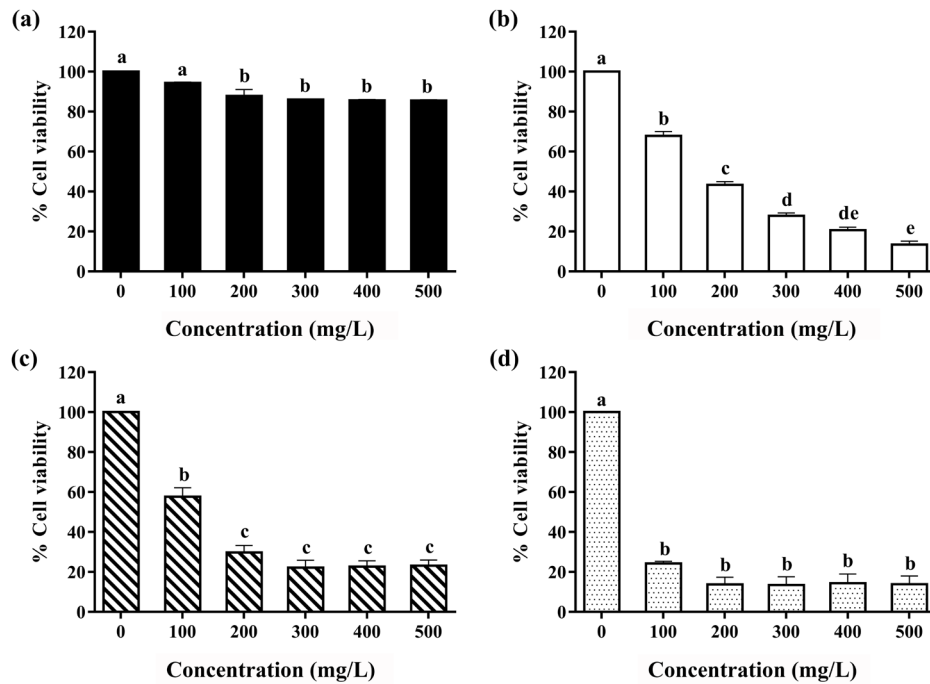


Figure 4. Dose-response curves of viability of koi carp PBMCs exposed to OCO at 10 min (a), 1 h (b), 6 h (c), and 12 h (d). Data are presented as means  $\pm$  S.E.M. ( $p < 0.05$ ).

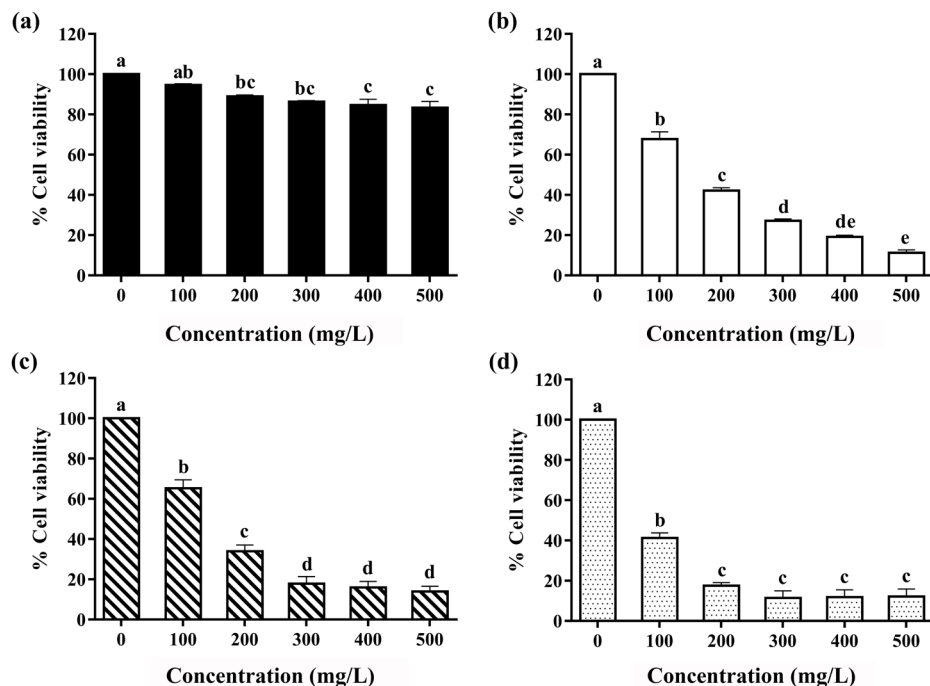


Figure 5. Dose-response curves of viability of koi carp PBMCs exposed to OSO at 10 min (a), 1 h (b), 6 h (c), and 12 h (d). Data are presented as means  $\pm$  S.E.M. ( $p < 0.05$ ).

environment conditions; geographic variations, genetic factors, and harvest period of plants (33). In the present study, chemical analysis of the EOs indicated that compounds existing in OBO contained no eugenol and very low amount of eugenol derivatives (0.44% methyl eugenol). Our study found that methyl chavicol was

the major constituent (78.12%) of OBO. This finding was in good agreement with the reports of Grayer *et al.* (34) and Hasegawa *et al.* (35). From the previous study, methyl chavicol showed the anesthetic activity in rats by direct inhibition of sodium channels, the major cause of excitability blockade (36). Therefore,

the fish anesthetic activity of OBO presented in the present study is considered to be mainly due to methyl chavicol.

OCO was previously reported to have different major compounds such as camphor (37,38), 1,8-cineole (39), citral (40), eugenol (41), geraniol (32), and linalool (42). However, in the present study, E-citral (41.01%) and Z-citral (37.04%) were found at the highest level in this EO. Even though, eugenol and its derivatives were not found, OCO showed the anesthetic activity in the fish. It was previously reported that E-citral and Z-citral had the anesthetic activity on white shrimps (*Litopenaeus vannamei*) (43) and Wistar rats (*Rattus norvegicus*) (44). Therefore, the fish anesthetic activity of OCO as found in the current study is considered to be due to these two isomers of citral. For OSO, eugenol was previously reported to be a major compound existing in this EO (19,45). In the current study, methyl eugenol (46.18%) was the most abundant. Eugenol was also existed (11.56%) but significantly lower amount than methyl eugenol. These results are similar to the findings of the other previous reports (46-48). Methyl eugenol has been reviewed to have anesthetic activity (49). Thus, the fish anesthetic effect of OSO is considered to be mainly due to methyl eugenol and the minor effect of eugenol. Among the three EOs, OSO showed the shortest induction time indicating the highest anesthetic activity, followed by OCO and OBO. The ideal anesthetic agent should meet the major criteria of high capability of rapid induction to surgical anesthesia within 3 or 5 min and the fish should recover within 5 min in anesthetic-free water. The recovery time should not be longer than two-fold of the induction time and no mortality should be occurred (50). Based on these criteria, the appropriated concentration for koi carp anesthesia of OBO, OCO, and OSO was 300, 200, and 100 mg/L, respectively.

An ideal fish anesthetic should also be non-toxicity to fish, low cost, causing rapid anesthesia without any side effects (50). The EOs used in the present study are easily extracted using simple distillation and the raw plant materials are easily available and low cost. All studied concentrations of OSO (100-300 mg/L) and 300 mg/L OCO showed a short induction time but very long recovery time. The three EOs showed longer recovery time than MS-222. This effects are considered to be due to the accumulation of lipophilic EOs in adipose tissue of the fish (51,52). The lipophilicity of plant EOs is higher than that of MS-222, therefore, the clearance and elimination of EOs was slower than MS-222.

Quantitative analysis of cell viability of fish PBMCs after contact with the EOs could indicate the safety or toxicity of the EOs to the fish. However, for direct contact of the fish PBMCs and the EOs, the EOs showed some toxic at different levels. It was found that the toxicity of OCO was almost same as OSO whereas OBO had significantly lower toxicity

on fish PBMCs than OCO and OSO, respectively. The secretion found in the fish exposed to OBO and OCO suggesting some minor side effects. Generally, it is regarded as safe when the cell viability is higher than 80% after exposure to the tested sample (53). The maximum induction time of all tested EOs was about 300 sec or 5 min. The results demonstrated that direct contact to the EOs up to 10 min, higher than 80% viability of the PBMCs was still obtained. Therefore, it could be confirmed that OBO, OCO, and OSO were safe for fish anesthesia. High viability of fish PBMC (> 80%) after exposed to 100 mg/L OBO for 1, 6, and 12 h, confirming that OBO was safer than the other two EOs. However, higher EO concentration and longer time of the direct exposure caused higher cytotoxicity to the cells. Because some previous studies have been reported that the whole blood concentrations of MS-222 in *Salmo gairdneri* (rainbow trout) was about 74% of the bath administration concentration after losing of reflex (54) and 70% of MS-222 in bath administration concentration was found in blood concentration of *Ictalurus punctatus* (catfish) (55). Therefore, our results suggested that it should be careful when high dose of these EOs were used for fish anesthesia particularly for a long period of anesthetized time for example in case of fish operation or long distance transportation.

In conclusion, our findings demonstrate that OBO, OCO, and OSO possess effective anesthetic activity to the fish. Their anesthetic efficacy are due to their respective main components; methyl chavicol for OBO, eugenol for OCO, and methyl eugenol and eugenol for OSO. The anesthetic activity and toxicity of OBO, OCO, and OSO is concentration and exposure time dependent. Using an effective dose within 10 min is confirmed for their anesthetic efficacy and safety. It is concluded that OBO, OCO, and OSO are novel promising natural anesthetics for fish.

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