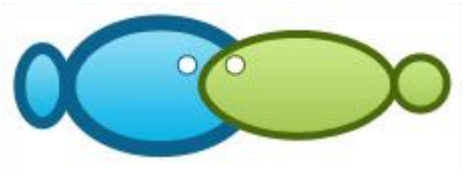

Anesthetic efficiency of *Spilanthes acmella* on anesthesia, haematocrit and histopathology of Nile tilapia *Oreochromis niloticus*

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Abstract. The anesthetic effects of *Spilanthes acmella* in Nile tilapia *Oreochromis niloticus* were determined. Nile tilapia with average 34.62±15.11 g were used in this study. Fishes were exposed to the extracts 1.0, 2.0, 3.0 and 4.0 mL L^{-1} compared with control group without the extracts. The results showed that the optimum concentration of the extracts were 2.0 mL L^{-1} which had the best effects in all parameters including induced anesthesia for 6 hours and could recover fish within 3.56±0.60 min, an average daily growth of 0.686±0.055 g day^{-1}, the percentage of haematocrit of 23.83±0.66 and no observed any abnormalities on histopathological finding of gills and liver tissues of Nile tilapia after exposed to the extracts. From these results, it can be concluded that *S. acmella* extracts had a high potential as a new anesthetics in Nile tilapia.
Key Words: anesthetic, extracts, *Oreochromis niloticus*, *Spilanthes acmella*.

Introduction. Aquaculture industries commonly induce stress condition in aquatic animals such as over stocking density, transportation and handing (Rehman et al 2017) or restraint due to vaccination. These can cause several problems to fish namely; slow growth, retardation and pathogen invasion (Ross & Ross 2008). Therefore, anesthetics play an important role to decrease these stress from aquaculture activities. The usage of anesthetics are dependent on the type of aquatic animals and anesthetics effectiveness (Rose 2002; Sneddon 2003; Chandroo et al 2004; Huntingford et al 2006; Sneddon 2012). The benefits of anesthetics are reduced injury, reduced oxygen demand during metabolism and reduced waste production (CO_2 and NH_3) (Cooke et al 2004; Hoskonen & Pirhonen 2004; Crosby et al 2006). The considered factor in inducing anesthesia was dependent on fish species and fish size, such as; the concentration of anesthetic was higher in larger fish than small fish. However, the larger fish showed quick response to anesthesia than smaller fish. Then, the duration for anesthesia was longer in larger or spawning fish as well as the duration of recovery. Moreover, the sick or weak fish during treatment was susceptible to anesthetics (Coyle et al 2004; Fernandes et al 2017).

Chemical anesthetics have been commonly used worldwide for example, MS-222 (TMS, tricaine methanesulfonate), benzocaine, metomidate, quinaldine and phenoxyethanol (Ross & Ross 2008). However, chemical anesthetics can induce side effects to the user after application such as; irritation after exposure, headache after ventilation, stagnant blood circulation and effects on the central nervous system (Durve 1966; Bell 1987). The residues of these chemicals are accumulated in fish tissues preventing these fish from human consumption. Thus, the alternative ways to use medicinal plants as anesthetics for reducing the usage of chemical agents were studied (Amani & James 2007).

Active ingredients from medicinal plants that have anesthetic effects have been reported as follows: clove (*Syzygium aromaticum*) as eugenol (Pirhonen & Schreck 2003; Mylonas et al 2005; Pattanasiri et al 2017), coca leaf (*Erythroxylum coca*) as cocaine, para cress (*Spilanthes acmella*) as spilanthol which is a N-isobutylamide compound

(Barbosa et al 2016), rubber seed (*Hevea brasilensis*) as alkaloid compound (Saputri et al 2019) and sweet flag (*Acorus calamus*) as acorin (Panchal et al 1989; Bhuvaneswari et al 2015). The alternative way to find a potential herbal extract anesthetic replacement to the use of chemical anesthetics is aimed in this research. In this study, *S. acmella*, commonly found in local areas in Thailand was used. There was a report that spilanthol is high in part of its flowers that contained 25.7% of it (Dias et al 2012). This is a first report of *S. acmella* extracts as anesthetics in Nile tilapia.

Material and Method

Experimental fish. The experiment was conducted from October to December, 2019. Nile tilapia (*Oreochromis niloticus*) with the average of 34.62±15.11 g and 13.15±0.86 cm were acclimatized for two weeks. Ten fish per tank were reared in 40 L of water and were fed twice a day at 5% body weight.

Preparation of Para cress extracts. Para cress (*S. acmella*) was collected from Muang District, Maha Sarakham Province. Briefly, para cress was washed, dried and ground into fine powder. One kilogram of para cress powder was soaked into 3 L of 95% ethanol and kept for 2 hours at 45ºC in water bath. Then, the extracts were filtrated through Whatman No. 1 then the solvents were evaporated using Rotary vacuum evaporator for 2 hours 30 min at 45ºC. Final extracts (8 mg L^{-1}) were adjusted to 10 mL with 95% ethanol and kept at 4ºC according to Leng et al (2011) and Pavunraj (2014).

Experiment trial I: efficacy of S. acmella extracts in Nile tilapia anesthesia. One hundred-fifty fish were divided into five groups with different concentrations of *S. acmella* extracts including 0, 1, 2, 3 and 4 mL L^{-1}. Each treatment was replicated three times with ten fish in each replication group. Anaesthetized fish were observed into four stages which modified from Coyle et al (2004) as follows:

- stage 0 = normal fish (no effect);
- stage 1 = lethargy, reduction of movement and breath (sedation);
- stage 2 = loss of equilibrium, no reaction to stimulator, operculum movement (anesthesia);
- stage 3 = no operculum movement (death).

The anesthetized fish behaviors were monitored every five minute for sixty minutes, and remained observed at 2, 4, 6, 12 and 24 hours. The recovery fish were determined after exposure to the extracts for 24 hours, then, these fish were reared for one week to investigate the optimum concentration for anesthetics application in Nile tilapia by monitoring the average daily growth of each group (Imjai et al 2018) by determining the average daily weight gained of experimental fish:

$$\text{Average daily growth (ADG)} = \frac{\text{Mean weight gained}}{\text{Length of feeding trial (days)}}$$

Experiment trial II: haematocrit of pre and post anesthetized fish. Six anesthetized fish were chosen in each concentration for blood collecting at caudal vein by using heparin as anticoagulant which slightly modified according to Blaxhall & Daisley (1973). Fish blood was taken into the capillary and then, centrifuged at 11,000 rpm min^{-1} for 5 minutes. Haematocrit of pre and post anesthetized fish was determined using NUVE NF 048 Micro & Haematocrit Centrifuge.

Experiment trial III: histopathological finding of Nile tilapia after exposure to S. acmella extracts. The anesthetized fish after exposure to several concentrations of *S. acmella* extracts were randomly collected for histopathological investigation (trial I). Briefly, gills and livers samples were fixed in 10% buffered formalin. Subsequently, the samples were routinely embedded in paraffin (Leica, Histocore Arcadia) and sectioned at 3 µm (Leica RM2125) according to Humason (1979). The sections were stained with hematoxylin and eosin according to Bancroft et al (1967).

Results

Efficacy of Spilanthes acmella extracts for Nile tilapia anesthesia. The results of *S. acmella* extract in Nile tilapia anesthesia were investigated into four concentrations including 0, 1, 2, 3 and 4 mL L^{-1}, respectively (Table 1). Nile tilapia was stayed at stage 0 until a period of experiment in control group. At concentration of 2 mL L^{-1}, fish was induced to stage 2 (70%) and stage 3 (30%) within 50 min after exposure. For other concentrations at 2, 3 and 4 mL L^{-1}, fish become stage 1 and 2 within 5 min, and remained at stage 2 for 6 hours. After exposed to the extracts at 2 and 3 mL L^{-1} for 8 hours, fish were recovered and were swimming normally. On the other hand, fish retained in stage 2 until 8 hours in the group of 4 mL L^{-1}, became normal.

Table 1

Stages of anesthesia of *O. niloticus* exposed to various concentrations of *S. acmella* extracts in static bioassay

Exposure time	*Stages of anesthesia after exposed to S. acmella extracts (mL L^{-1})*				
	Control	*1.0*	*2.0*	*3.0*	*4.0*
5 min	0	0	1 (10%), 2 (90%)	2	2
10 min	0	0	2	2	2
15 min	0	0	2	2	2
20 min	0	0	2	2	2
25 min	0	0	2	2	2
30 min	0	1	2	2	2
35 min	0	1	2	2	2
40 min	0	1	2	2	2
45 min	0	1	2	2	2
50 min	0	1(70%), 2(30%)	2	2	2
55 min	0	1(70%), 2(30%)	2	2	2
1 hours	0	1(80%), 2(20%)	2	2	2
2 hours	0	0	2	2	2
4 hours	0	0	2	2	2
6 hours	0	0	1(13.33%), 2(86.67%)	1(10%), 2(90%)	2
8 hours	0	0	0	0	2
12 hours	0	0	0	0	0
18 hours	0	0	0	0	0
24 hours	0	0	0	0	0

Stages of anesthesia : 0 = no effect, 1 = sedation, 2 = anesthesia, 3 = death. The percentage in parentheses indicates the fish was induced anesthesia in stages 1 and 2.

The recovery stage of fish after exposed to *S. acmella* extracts concentrations at 1.0, 2.0, 3.0 and 4.0 mL L^{-1} were 2.22±0.25, 3.56±0.60, 3.93±0.40 and 4.56±0.40 min, respectively (Table 2). All fish can recover within 5 minutes in each concentration before the end of the experiment.

Table 2

The recovery stage of fish after exposed to *S. acmella* extracts

S. acmella extracts concentrations (mL L^{-1})	*Period of recovery stage (min)*
1.0	2.22 ± 0.25^{c}
2.0	3.56 ± 0.60^{b}
3.0	3.93 ± 0.40^{ab}
4.0	4.56 ± 0.40^{a}

Mean values in columns with differnet superscripts are significantly different ($p < 0.05$).

Average daily growth of fish after exposed to *S. acmella* extracts at 0, 1.0, 2.0, 3.0 and 4.0 mL L^{-1} were 0.340±0.010, 0.326±0.251, 0.686±0.055, 0.433±0.051, 0.376±0.005 g d^{-1}, respectively. Moreover, overage length were 0.040±0.010, 0.060±0.010, 0.080±0.010, 0.060±0.020 and 0.040±0.010 cm d^{-1}, respectively. The highest growth performance was observed at 2 mL L^{-1} (Table 3).

Table 3

Average daily growth of Nile tilapia after exposure to the various concentration of *S. acmella* extracts

S. acmella extracts concentrations (mL L^{-1})	*ADG (g d^{-1})*	*Leght (cm d^{-1})*
0	$0.340±0.010^{c}$	$0.040±0.010^{b}$
1.0	$0.326±0.251^{c}$	$0.060±0.010^{a}$
2.0	$0.686±0.055^{a}$	$0.080±0.010^{ab}$
3.0	$0.433±0.051^{b}$	$0.060±0.020^{ab}$
4.0	$0.376±0.005^{bc}$	$0.040±0.010^{b}$

Mean values in columns with differnet superscripts are significantly different ($p < 0.05$).

Haematocrit of pre and post anesthetized fish. The results showed the percentages of haematocrit were not significantly different among the experimental groups in pre anesthetized fish. On the other hand, percentages of haematocrit of post anesthetized fish were increased in all experimented groups, except the group exposed to 2.0 mL L^{-1} which was decreased. The highest percentage was in case of the concentrations of 3 and 4 mL L^{-1} (Table 4).

Table 4

Haematocrit of Nile tilapia after exposure to the various concentration of *S. acmella* extracts

S. acmella extracts concentrations (mL L^{-1})	*Haematocrit*		
	Pre anaesthetized fish	*Post anaesthetized fish (30 min)*	*Post anaesthetized fish (1 wk)*
0	$20.60±1.21^{Ba}$	$20.66±0.28^{Bb}$	$21.16±1.04^{Ab}$
1.0	$20.61±0.78^{Ba}$	$20.76±0.25^{Bb}$	$23.66±0.76^{Aa}$
2.0	$21.16±0.16^{Ba}$	$21.10±0.45^{Bab}$	$23.83±0.66^{Aa}$
3.0	$21.55±0.58^{Ba}$	$21.66±0.28^{Ba}$	$23.66±0.76^{Aa}$
4.0	$21.22±0.48^{Ba}$	$21.43±0.40^{Ba}$	$24.66±0.57^{Aa}$

Mean values in columns with different small letter superscripts and capital letter in rows with different small letter superscripts are significantly different ($p < 0.05$).

Histopathological finding of Nile tilapia after exposure to S. acmella extracts. In total six fish were randomly collected in each group for histopathological examination. Gills of control group and fish exposed to *S. acmella* extracts at 1.0 and 2.0 mL L^{-1} showed normal appearance of gill filaments and gill lamellae (Figure 1A). On the other hand, fish exposed to 3.0 and 4.0 ml L^{-1} slightly changed its gills such as epithelial lifting (EL) (Figure 1B). The liver tissue of fish exposed to *S. acmella* extracts at 1.0, 2.0 and 3.0 mL L^{-1} showed normal characteristics compared to the control group (Figure 2A). However, fish exposed to 4.0 mL L^{-1} mildly changed such as degeneration of hepatocytes and hepatocyte cytoplasmic vacuolation (Figure 2B).

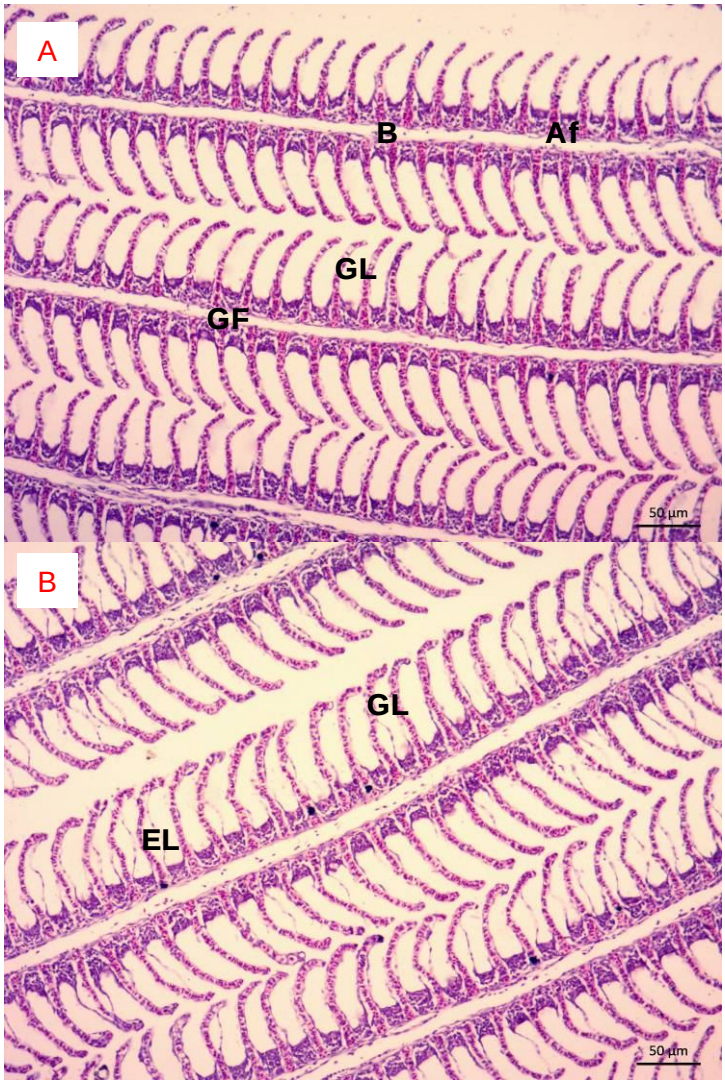

Figure 1. Histopathological section of gills of Nile tilapia after exposed to the *S. acmella* extracts (H&E, 50 µm). (A) Control group showing normal feature of gill lamellae; (B) Fish exposed to *S. acmella* extracts at 4.0 mL L^{-1} showing epithelial lifting (EL) in gill lamellae. GF: gill filaments, GL: gill lamellae, Af: afferent arteriole, B: blood cells.

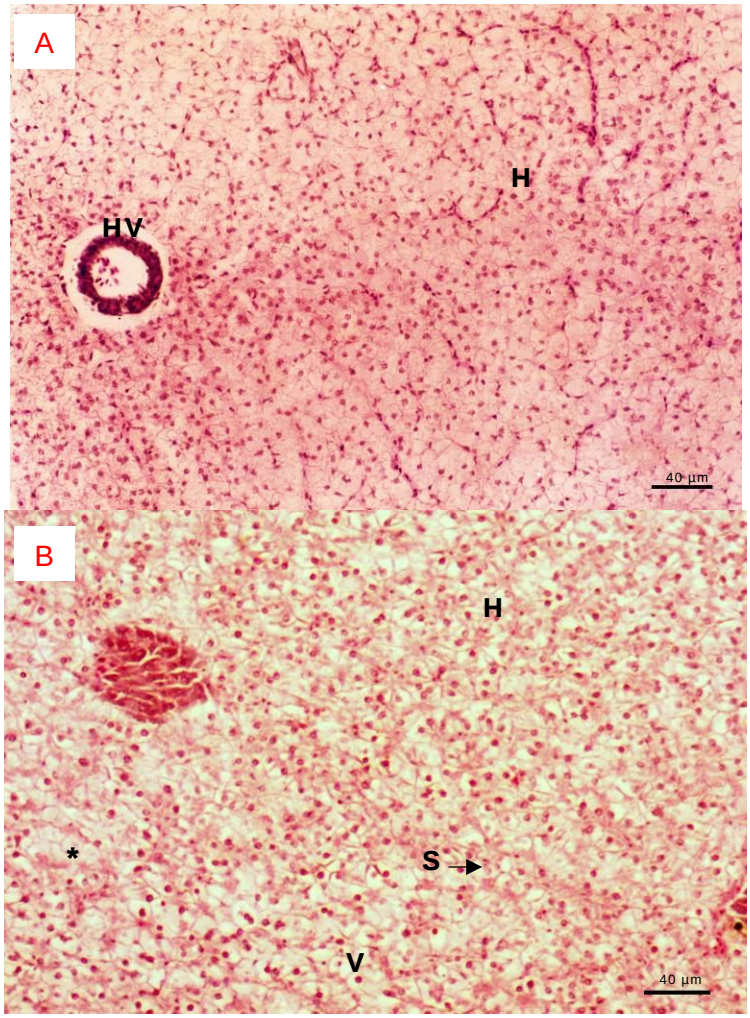

Figure 2. Histopathological section of liver of Nile tilapia after exposed to the *S. acmella* extracts (H&E, 40 µm). (A) Control group showing normal appearance of hepatocytes; (B) Fish exposed to *S. acmella* extracts at 4.0 mL L^{-1} showing degeneration of liver tissue (*) and hepatocyte cytoplasmic vacuolation. H: hepatocyte, S: sinusoid, HV: hapatic vein, V: vacuoles.

Discussion. The optimum concentration of *S. acmella* extracts on Nile tilapia anesthesia was 2.0 mL L^{-1} which induced anesthetized fish into stage 1 and 2 within 3.56±0.60 min. From these results, *S. acmella* extracts can be an effective fish anesthetic since the extracts can induce anesthesia to fish within 1 to 5 minutes only. Moreover, fish can recover in less than 5 minutes. Factors affecting the efficacy of fish anesthesia were dependent on types and doses of anesthetics namely; easy and safe to use, no effects to fish physiology and behavior, rapid excretion out from the body and the lack of any residues into the fish tissues (Rose 2002; Sneddon 2003; Chandroo et al 2004; Coyle et al 2004; Huntingford et al 2006; Browm 2011; Sneddon 2012). In this study, the *S. acmella* extracts could be induced to fish into stage 2 of anesthesia for 6 hours which is in contrary to the report of Barbas et al (2016) who studied in juvenile tambaqui with *S. acmella* as anesthetics at concentration of 20 mg L^{-1}. The extraction technique of Barbas et al (2016) used supercritical fluid extraction with final yields at 834 g L^{-1}, nevertheless in the present study, 95% ethyl alcohol was used as a solvent which was an easy method for application in fish.

Therefore, the discrimination of anesthetics was dependent on type of selected aquatic animals wherein the larger aquatic animals had to use higher doses than smaller aquatic animals. On the other hand, larger aquatic animals had higher responses to induced anesthesia than smaller aquatic animals. The duration of anesthesia was longer in case of larger or spawning fish as well as the duration of recovery (Coyle et al 2004).

Moreover, the sick or weak fish during treatment was susceptible to anesthetics (Coyle et al 2004). The chemical anesthetics have been commonly used worldwide such as MS-222 (TMS, tricaine methanesulfonate), benzocaine, metomidate, quinaldine and phenoxyethanol (Ross & Ross 2008). The residues of these chemicals were accumulated in fish tissues and some anesthetics were prohibited for fish rearing for human consumption (Bell 1987; Burridge et al 2010). Furthermore, chemical anesthetics can induce abnormal effects to the users after application such as irritation after exposure, headache after ventilation, stopped blood circulation and effects on central nervous system (Neiffer & Stamper 2009). Thus, the advantage of alternative medicinal plants anesthetics was worthy for reducing the chemicals residues and side effects in aquatic animals as well as users (Amani & James 2007).

Haematocrit was not significantly different among the experimental groups ($p > 0.05$). The haematocrit was increased after reared for 1 week and its haematocrit value corresponded to the variance of average daily growth which showed a normal value at 15-45% (Bittencourt et al 2003).

Histopathological changes of Nile tilapia after exposure to *S. acmella* extracts for 1 week were determined. The microscopic features of gill tissues showed normal appearance at 0, 1.0 and 2.0 mL L^{-1}. The abnormal characteristics such as epithelial lifting in gills lamellae were found at 3.0 and 4.0 mL L^{-1}. These abnormal findings described physiological changing of fish gills for reducing influxes of toxic substances penetrated into other organs after exposure to hazards (Perry & Laurent 1993) and this changing could indicate the harmful effect of aquatic environments (Flores-Lopes & Thomaz 2011). No histopathological changes were found in liver tissues at concentration of 0, 1.0, 2.0 and 3.0 mL L^{-1}, but the presence of cell necrosis and vacuolated cytoplasm in hepatocytes was observed at 4.0 mL L^{-1}. Hepatocyte cytoplasmic vacuolation associated with glycogen accumulation in hepatocytes or the glycogen depletion could be aroused during the preparation of permanent slides (Grizzle & Roger 1976).

Conclusions. The effectiveness of *S. acmella* extracts as anesthetics in Nile tilapia was performed in which the optimum concentration as 2 mL L^{-1} could be induced anesthesia for 6 hours, duration of fish recovered at 3.56±0.60 min and haematocrit valued at 23.83±0.66%. No histopathological changings were observed in gills and liver of anesthetized fish at 2.0 mL L^{-1}. From these results, it can be concluded that *S. acmella* extracts had a high potential for choosing as a new anesthetics in Nile tilapia.

Acknowledgements. Authors with to thanks faculty of Agricultural Technology, Rajabhat Maha Sarakham University for providing necessary facilities for the research. Special Thanks to Food Technology program for preparing herb extracts and Assist. Prof. Dr. C. Kanchan Aquaculture Technology Program for moral supports.

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Received: 26 October 2020. Accepted: 17 January 2021. Published online: 08 March 2021.
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How to cite this article:
Imjai P., Rujinanont N., Gawborisut S., Srisakultiew P., 2021 Anesthetic efficiency of *Spilanthes acmella* on anesthesia, haematocrit and histopathology of Nile tilapia *Oreochromis niloticus*. AACL Bioflux 14(2):695-703.