*Short Communication*

# Toxicity Test of Kameng (*Eclipta prostrate* Linn.) and Kradhuawean (*Spilanthes acmella* (Linn.) Murr.) to Early Life Stage of Zebrafish (*Danio rerio*)

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***Abstract***

The toxicity of Kameng (*Eclipta prostrate* Linn.) and Kradhuawean (*Spilanthes acmella* (Linn.) Murr.) on zebrafish (*Danio rerio*) embryos was investigated. An aqueous crude extract of the leaves of each plant was tested with newly spawned embryos in 96 well plates. The observed effect was based on the toxicity endpoint composed of lethal, sublethal and malformation effects appearing at 24, 48 and 144 hours post-fertilization (hpf). The results suggest that the lowest observed effect concentrations (LOEC) for a lethal and sublethal effect of *Eclipta prostrate* Linn. at 48 hpf were 1% and 0.1% while the no observed effect concentrations (NOEC) for the lethal and sublethal effects of the plants at 48 hpf were 0.01% and 0.1%, respectively. LOEC for malformation and sublethal effects of *Spilanthes acmella* Linn. Murr. at 48 hpf were 20% and 10% while NOEC for lethal, malformation and sublethal effects of *S. acmella* Linn. Murr. at 48 hpf were 20%, 10% and 1%, respectively. There was no lethal effect observable in the highest concentration test to the embryo at 20% of *Spilanthes acmella* Linn. Murr.

**Keywords:** *Danio rerio, Eclipta prostrate* Linn., *Spilanthes acmella* Linn. Murr., toxicity test

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# บทคัดย่อ

## การทดสอบความเป็นพิษของกะเม็ง (*Eclipta prostrate* Linn.) และคราดหัวแหวน (*Spilanthes acmella* (Linn.) Murr.) ต่อตัวอ่อนปลาม้าลาย (*Danio rerio*)

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การศึกษาความเป็นพิษของสารสกัดหยาบใบกะเม็ง (*Eclipta prostrate* Linn.) และคราดหัวแหวน (*Spilanthes acmella* (Linn.) Murr.) ต่อตัวอ่อนภายในไข่ของปลาม้าลาย (*Danio rerio*) โดยการทดสอบในจานหลุมแบบ 96 ช่อง ทดสอบความเป็นพิษในระดับต่างๆ และผลต่อพัฒนาการของตัวอ่อนที่เวลา 24 ชั่วโมง 48 ชั่วโมงและ 144 ชั่วโมงภายหลังจากไข่ได้รับการผสม ผลการศึกษาพบว่าความเข้มข้นต่ำที่สุดร้อยละ 1 ของสารสกัดหยาบใบกะเม็ง มีผลทำให้ตัวอ่อนตายที่เวลา 48 ชั่วโมง ค่าความเข้มข้นสูงสุดที่ ร้อยละ 0.01 ไม่พบความผิดปกติใดๆ ค่าความเข้มข้นต่ำที่สุด ร้อยละ 10 ของสารสกัดหยาบใบคราดหัวแหวน ทำให้ตัวอ่อนเกิดความผิดปกติที่เวลา 48 ชั่วโมง ค่าความเข้มข้นที่สูงสุดร้อยละ 1 ไม่พบความผิดปกติใดๆ ทั้งนี้ ไม่พบว่าสารสกัดหยาบใบคราดหัวแหวนทำให้ตัวอ่อนตายในระดับความเข้มข้นที่ทำการทดสอบไม่เกินร้อยละ 20

**คำสำคัญ:** *Eclipta prostrate* Linn. *Spilanthes acmella* Linn. Murr. ปลาม้าลาย ทดสอบความเป็นพิษ

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## *Introduction*

Kameng or false Daisy (*Eclipta prostrate* Linn.) and Kradhuawean or para cress (*Spilanthes acmella* (Linn.) Murr.) are members of a herbaceous plant in the Asteraceae family. They are non-ingested weeds that commonly appear in cattle pastures, but people used them as medicinal herbs. In Asia, *S. acmella* has been known as a toothache plant because of its analgesic activity produced by bioactive compounds such as spilanthal (Nakatani and Nagashima, 1992) and flavonoid (Chakraborty et al, 2004). It has been used for stomatitis and throat complaints (Nakatani and Nagashima, 1992). *E. prostrate* has been used as a traditional medicine to treat hyperlipidemia, atherosclerosis, hepatic disorders, skin diseases (Kim et al, 2008). Isolation of *E. prostrate* aerial part extract provided a phytosterol including triterpenoids (Prachayasittikul et al., 2010). Its aqueous extract has been found to have inhibiting activity on the invasion of HCC-S102 liver cancer cells (Lirdprapamongkol et al., 2008) and mosquito larvicidal activity (Elango et al., 2009). In our previous study we found that *E. prostrate* Linn. and *Spilanthes acmella* Linn. Murr. contained high yields of testosterone, *E. prostrate* contained 0.59 ng/g dry weight, and *S. acmella* contained 1.39 ng/g dry weight (Suthikrai et al., 2010). Therefore, they have the potential to be used as medicinal herbs or as animal hormonal feed supplements. To investigate the toxicity of the plants on humans and animals before consumption, one useful and highly sensitive screening test is to expose the plant extract to the early life stage of zebrafish (*Danio rerio*) or the fish embryonic toxicity test which is established (OECD 1992; ISO 1999; Carlsson and Norrgren, 2004). The test is one of the most widely used tools in environmental science research, especially for investigating the toxicity and teratogenicity of chemicals that could significantly affect environmental health (Xiaoshan et al., 2007). In many studies, *D. rerio* has been used as a vertebrate model to study the developmental toxicity of chemical compounds and has contributed to the understanding of the potential toxicological and ecotoxicological impact on the human and aquatic environment (Laale, 1977; Carlsson and Norrgren, 2004; Incardona et al., 2004; Dagmara et al., 2005; Xiaoshan et al., 2007). *D. rerio* is a freshwater tropical species, native to India and Pakistan, and a member of the cyprinidae family (Laale, 1977). It is beneficial as a test species since it is small in size, robust, easy to maintain and has a short generation time. Zebrafish are oviparous with transparent, non-adherent eggs. At 24 hour the embryo has developed eyes and a tail. At 48 hour, pigmentation can be seen on the eyes and body. The heart is developed and circulation can also be observed. It is possible to count the heartbeat and

thereby determine the heart frequency. The embryo undergoes a fast development and the larvae hatches in 96 hours at 26$^0$C (Laale, 1977). However, there is no reported study of the *D. rerio* embryonic toxicity test in *E. prostrate* and *S. acmella* therefore we would like to investigate the toxic effect of crude extract of *E. prostrate* and *S. acmella* on *D. rerio* embryo to expand the future use of medicinal plant in human and animals.

## *Materials and Methods*

The standardized water used throughout the experiments was prepared from deionized water, with the following salts added: $CaCl_2$ x $2H_2O$ (117.6 mg/l), $MgSO_4$ x $7H_2O$ (49.3 mg/l), $NaHCO_3$ (25.9 mg/l), and KCl (2.3 mg/l) (ISO 1996). The water was continuously aerated at least overnight before use. The salts for the standardized water were obtained from Fisher Scientific. The concentration of crude extract in the stock solutions was $10^4$ times higher than the nominal exposure concentrations of the tested compounds. The exposure concentrations were obtained by mixing the stock solution with standardized water, resulting in a concentration of 0.01- 40%.

***Crude extract preparations:*** The fresh leaves of Kameng (*Eclipta prostrate* Linn.) and Kradhuawean (*Spilanthes acmella* Linn.) were collected in August 2008 from Chachoengsao province in the eastern part of Thailand and authenticated by comparison with the herbarium specimens from department of botany. The leaves of those two plants were dried in hot air oven at 40$^0$C (moisture 26.0%). The dried sample was ground to powder. Powder from the leaves was kept at 6$^0$C in a refrigerator until used. Modification method from the method of Gailliot (1988) was used to obtain the leaf extract. 0.1 mg of dried leaf powder was extracted with deionizer water at 40$^0$C and vigorously stirred for one hour. The extraction sample was then filtered through a 0.4 µM diameter membrane filter. Final crude extraction was 1,000 ppm (mg/l) and was kept as stock solution for the following test at 6$^0$C until used.

***Fish embryo collection:*** A hundred mature male and female zebrafish were kept together in a 200 litre glass aquarium supplied with carbon filtered water. They were fed frozen tubifex worms in the morning and dried tubifex or Tetramin flakes in the afternoon. They were cared for in a room that allowed exposure to natural light and dark hours. The day before the embryonic toxicity test was to be performed, 20-25 individuals, a mixture of males and females zebrafish, were selected and transferred to stainless steel spawning cages in a glass aquarium with 4 l of carbon filtered water. After mating, the eggs fell through the cage to the bottom of the tank. The brood fish were, thereafter, returned to their original aquariums and the eggs were collected.

***Embryo toxicity study:*** This assay is basically the method described by Carlsson and Norrgren (2004). To start the exposure as soon as possible, the eggs were divided into 6 groups and put immediately into 100 ml beakers containing 50 ml of each of the test dilutions including the standard water control group. Fertilized eggs which had reached at least the 4 cell embryo stage were transferred individually to wells of 96-well plates including 250 ml of diluted crude extract or standard water rearing media which was not changed during the study. The treatment concentrations were prepared on the same day as the test to minimize the dilution effect. Twelve embryos were tested for each concentration. Six concentrations (0.01, 0.1, 1, 10, 20, 40%) of *E. prostrate* Linn and five concentrations (0.01, 0.1, 1, 10, 20%) of *S. acmella* Linn. Murr. and a standard water control test of each group were employed. All treatments consisted of 2 replicates. The well plates were covered with parafilm and placed in a temperature controlled room at 28$^0$C. Observations of the accumulation of lethal, sublethal (reversible side effect) and malformation (nonreversible side effect) endpoints were made with a stereomicroscope at 24, 48, and 144 hours post-fertilization (hpf). The parameters observed at 24 hpf were coagulation, full eye development, somite formation, full tail extension and body movement. The parameters observed at 48 hpf were coagulation, full eye development, somite formation, full tail extension, heart beat, edema, congestion, body circulation and pigmentation. The parameters observed at 144 hpf were hatching and abnormal body curvature. Lethal endpoints included coagulation and lack of heartbeat. Sublethal endpoints included the presence of edema, congestion, lack of body circulation, not fully developed pigmentation. Malformation endpoints included not full eye development, not full tail extension, lack of body movement and non-hatching at 144 hpf. A time-lapse camera photographed the well plates at 1-hour intervals between 48 and 144 hpf. The number of heart beats per minute was observed individually. The photographs were examined visually to determine the hatching time for each individual. The lowest observed effect concentrations (LOEC) and the no observed effect concentrations (NOEC) at 48 hpf were recorded.

**Table 1** Description of endpoints lowest observed effect concentrations (LOEC) and no observed effect concentrations (NOEC) at 48 hpf in the zebrafish embryo test for Kameng (*E. prostrate* Linn.) and Kradhuawean (*S. acmella* (Linn.) Murr.)

| Endpoints | LOEC (%) | | NOEC (%) | |
|---|---|---|---|---|
| | *E. prostrate* | *S. acmella* | *E. prostrate* | *S. acmella* |
| Lethal | 1 | - | 0.01 | 20 |
| Sublethal | 0.1 | 10 | 0.1 | 1 |
| Malformation | - | 20 | - | 10 |
| Lethal or sublethal or malformation | 0.1 | 10 | 0.01 | 1 |

## *Results and Discussion*

*Eclipta prostrate* Linn. and *Spilanthes acmella* (Linn.) Murr. are natural plants commonly grown in pasture and have been used as herbs for many purposes to improve human health. In our previous study we found that these two kinds of plant contained a high yield of phytotestosterone 0.59-1.39 ng/g dry weight (Suthikrai et al, 2010). While serum testosterone in pubertal development rams was 0.6-3.1 ng/ml (Schanbacher, Crouse and Ferrell, 1980). Testosterone has an influence on growth rate and feed utilization in low doses-dependent variation in sheep and cattle (Casida et al, 1959; Schanbacher, Crouse and Ferrell, 1980). It also has an influence on carcass characteristics by reducing back fat thickness (Schanbacher, Crouse and Ferrell, 1980). Although utilization of these non-ingested plants in the pasture can be beneficial to animal, the toxicity of the plant to animal and environmental effect should be concerned. The present study used the zebrafish embryonic toxicity test method (FET) which has been established for environmental toxicological study (OECD, 1992; ISO, 1999), to investigate the toxic effect of crude extract of *E. prostrate* Linn and *S. acmella* (Linn.) Murr.. Because the zebrafish embryo test is a highly sensitive toxicity test of chemical substances on animals, the result can be used as basic data for the toxicity test in higher animals and environmental contamination regulation. The results are presented in table 1 and Figs 1-4 which show that the lowest observed effect concentrations (LOEC) for lethal and sublethal effects of *E. prostrate* Linn. were 1% and 0.1% while no observed effect concentrations (NOEC) for lethal and sublethal effects of the plant were 0.01% and 0.1%, respectively. For *S. acmella* (Linn.) Murr., there was no lethal effect on zebrafish embryo at 20% v/v, which was the highest concentration test of the study, while significantly lowest observable sublethal effect concentration was 10%. All sublethal effect response embryos had delayed developed pigmentation compared to the control group. At 20%, malformation effects consisted of incomplete development of the eye and tail at 48 hpf and non-hatching at 144 hpf was present. According to this study, crude extract of *E. prostrate* Linn. and *S. acmella* (Linn.) Murr. can be used in animal feed at 0.01% v/v and 1% v/v, respectively, without any lethal, sublethal and malformation effect.

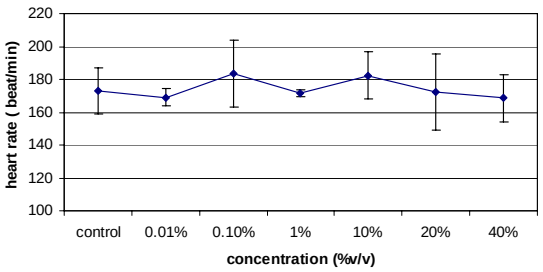


**Figure 1** Heart rate of the zebrafish embryo test for Kameng (*E. prostrate* Linn.) has no statistically significant difference between different concentrations.

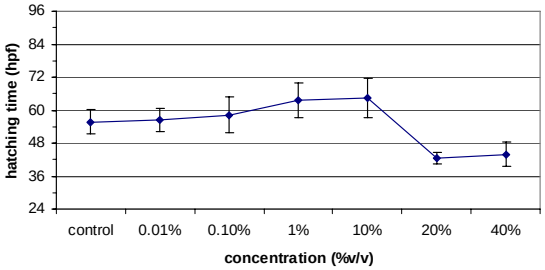


**Figure 2** Hatching time of the zebrafish embryo test for Kameng (*E. prostrate* Linn.). Although there is no statistically significant difference between different concentrations, the higher concentration seems to stimulate hatchability, thus reducing the hatching time.

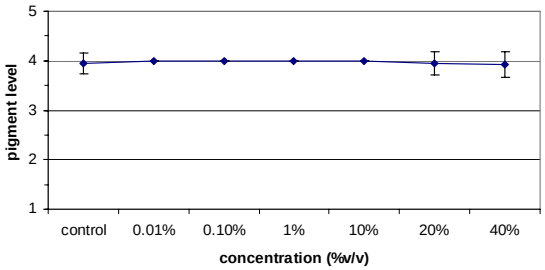


**Figure 3** Pigment level of the zebrafish embryo test for Kameng (*E. prostrate* Linn.) has no statistically significant difference between different concentrations.

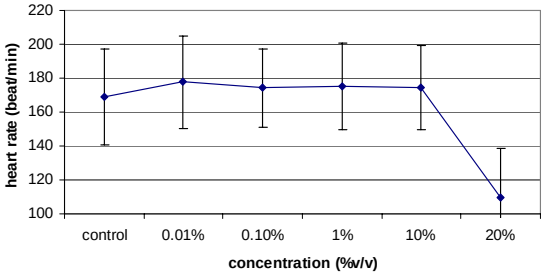


**Figure 4** Heart rate of the zebrafish embryo test for Kradhuawean (*S. acmella* (Linn.) Murr.) Although there is no statistically significant difference between different concentrations, the higher concentration seems to decrease the heart rate.

## *Acknowledgements*

This study was financially supported by Chulalongkorn University Centenary Academic Development Project and Faculty of Veterinary Science Innovation Center grant No 06/53.

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