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Effect of Wild Cape Gooseberry (Physalis minima Linn.) on Zebra fish (Danio rerio) Embryo

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Short Communication

Effect of Wild Cape Gooseberry (*Physalis minima* Linn.) on Zebra fish (*Danio rerio*) Embryo

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Abstract

The effect of crude extract of wild cape gooseberry, *P. minima* Linn. on zebra fish, *D. rerio*, embryo was investigated. Five concentrations (0.01%, 0.1%, 1%, 10% and 20%) of an aqueous crude extract of the plant leaf was prepared and tested with newly spawned embryos in 96 well plates. The observation effect was based on the toxicity endpoint composed of lethal effects, sublethal effects and malformation effects appearing within 144 hours post-fertilization (hpf). The results suggested that the lowest observed effect concentrations (LOEC) for a lethal effect and a sublethal effect were 20% and 10% respectively. Not fully developed pigmentation of the embryo and bradycardia were the sublethal effects found in this study at 10% and 20% concentrations. There was no observable malformation effect in any of the test concentration. The no observed effect concentrations (NOEC) for a lethal effect and a sublethal effect were 10%, and 1% respectively.

Keywords: *Danio rerio, Physalis minima* Linn., toxicity test

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บทคัดย่อ

การทดสอบความเป็นพิษของใบโทงเทง (*Physalis minima* Linn.) ต่อตัวอ่อนปลาม้าลาย (*Danio rerio*)

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ศึกษาความเป็นพิษของสารสกัดหยาบใบโทงเทง (*P. minima* Linn.) ต่อตัวอ่อนปลาม้าลาย (*D. rerio*) ที่ระดับความเข้มข้นร้อยละ 0.01, 0.1, 1, 10 และ 20 ของสารสกัดจากใบดังกล่าว โดยทำการทดสอบในจานหลุมแบบ 96 ช่อง ทดสอบความเป็นพิษในระดับตาย (lethal effect) ระดับต่ำกว่าตาย (sublethal effect) และระดับพิการ (malformation effect) ภายในเวลา 144 ชั่วโมงหลังจากไข่ได้รับการผสม พบว่าความเข้มข้นที่ต่ำที่สุดของสารสกัดหยาบใบโทงเทงที่มีผลต่อตัวอ่อน (LOEC) ระดับตาย เท่ากับร้อยละ20 และระดับต่ำกว่าตายเท่ากับร้อยละ10 โดยความผิดปกติระดับต่ำกว่าตายที่พบ คือหัวใจเต้นช้าและพัฒนาการของเม็ดสีบนลำตัวน้อยที่ความเข้มข้นร้อยละ 10 และ 20 และไม่พบความผิดปกติแบบพิการในทุกระดับความเข้มข้นของสารสกัดหยาบที่ทดสอบ สำหรับความเข้มข้นสูงสุดที่ไม่มีผลต่อตัวอ่อน (NOEC) ระดับตาย และระดับต่ำกว่าตายเท่ากับร้อยละ 10 และ 1 ตามลำดับ

คำสำคัญ: *Physalis minima* Linn. ปลาม้าลาย การทดสอบความเป็นพิษ

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Introduction

Wild cape gooseberry or Tongteng, *Physalis minima* Linn., is a member of a herbaceous plant in the Solanaceae family widely used in folk medicine. The plant is a non-ingested weed that commonly appears in cattle pastures. It is an annual plant that grows up to 50 cm with a single egg shaped type leaf. The flower has a greenish yellow color appearing between the leaves. The fruit has a round shape of about 1 cm in diameter and is yellowish when ripe. In our previous study we used radioimmunoassay (RIA) technique to measure progesterone level in crude extracts of the air-dried leaf and we found 0.28 ng/ml progesterone in diethyl ether extraction (Suthikrai et al., 2010). Progesterone is a natural mammalian gonadal hormone produced by corpus luteal in the ovary. The major function of this hormone is to prepare endometrium for pregnant animals and reduce uterus contractions during pregnancy. In Veterinary medicine, progesterone is widely used for estrous suppression by inhibiting follicular development and preventing ovulation in animals. In general, the level of progesterone in canine serum during proestrus is 0.2-0.4 ng/ml (Concannon et al., 2009) while in dairy cows, plasma progesterone concentration before ovulation is about 2 ng/ml (Roelofs et al., 2006). Thus, the yield obtained from P. minima Linn. extraction is very high compared to the canine and dairy cow serum progesterone level. Plant progesterone has been revealed in a variety of plants such as the leaves of walnut trees, *Juglans regia* (Pauli et al., 2010), the shoots of peas, *Pisum sativum* and the shoots of mouse ear cress, *Arabidopsis thaliana* (Mayumi et al., 2007). However, a plant leaf always contains more than a single chemical compound, so before studying in detail other chemical compounds with costly and complicated techniques we can use simple techniques to investigate the toxicity of the plant in order to demonstrate the possibility of the animal application (Ponpornpisit et al., 2011). One useful and highly sensitive screening toxicity test is the FET (Fish Embryonic Toxicity) test (OECD 1992; ISO 1999; Carlsson and Norrgren, 2004; Ponpornpisit et al., 2011). The test is one of the most widely used tools in environmental science research, especially for investigating the toxicity and teratogenicity of chemicals that can significantly affect environmental health (Xiaoshan et al., 2007). It is rapid, quantitative and reproducible with only small amounts of chemicals being needed. 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 potential toxicological and ecotoxicological impact on human and aquatic environment (Laale, 1977; Carlsson and Norrgren, 2004; Incardona et al., 2004; Dagmara et al., 2005; Xiaoshan et al., 2007; D'amico et al., 2011). *D. rerio*, a member of the cyprinidae family, is a freshwater tropical species native to India and Pakistan (Laale, 1977) that is beneficial as a test species because of its small size, short generation time and transparent non-adherent eggs. At 24 hours the embryo has developed eyes and a tail. At 48 hours, pigmentation can be seen in the eyes and body. The

heart is developed and circulation can also be observed. It is possible to count the heartbeat and consequently determine the heart rate. The embryo undergoes a fast development and the larvae hatches in 96 hours at 26^0C (Laale, 1977). It is considered to be a replacement species for higher vertebrates for the study of toxicology. In this study, we want to investigate the toxic effect of crude extract of *P. minima* Linn. on *D. rerio* embryo to expand future application of medicinal plants in humans and animals.

Materials and Methods

The standardized water used throughout the experiments was prepared from deionized water, with the following salts added: $CaCl_2x2H_2O$ (117.6 mg/l), $MgSO_4x7H_2O$ (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 standardized water is used to expose the fish embryo in control group.

Crude extract preparations: Fresh leaves of wild cape gooseberry (*P. minima* Linn.) were collected in August 2008 from Chachoengsao province in the eastern part of Thailand and authenticated by comparison with the herbarium specimens from the Department of Botany, Faculty of Science, Chulalongkorn University. The leaves were dried in a hot air oven at 40^0C (Moisture 26.0%). The dried sample was ground to powder. Powder from the leaves was kept at 6^0C in a refrigerator until use. The modification method from Gailliot (1988) was used to obtain the leaf extract (Ponpornpisit et al., 2011). A 1,000 ppm (mg/l) crude extract was prepared from 10 mg of the dried leaf powder in 10 ml of deionized water at 40°C and vigorously stirred for one hour. The extraction sample was then filtered through a 0.4 µM diameter membrane filter. The final crude extraction was kept as the stock solution for the following test at 6^0C until use. The exposure concentrations were obtained by mixing the stock solution with standardized water to get five concentrations of 0.01%, 0.1%, 1%, 10% and 20% of the crude extract.

Fish embryo collection: A hundred mature male and female zebrafish were kept together in a 200 l glass aquarium supplied with carbon filtered water. They were fed frozen tubifex worms in the morning and dried tubifex (Hikari, Kyorin Co, Ltd) or flakes (Tetramin, Tetra Holding GmbH) in the afternoon. They were cared for in a room that allowed exposure to natural light and darkness. On the day before the embryonic toxicity test was to be performed, 20-25 individuals, a mixture of male and female zebra fish 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 was 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 solutions including the standard water as a control group. Fertilized eggs which had reached at least the 4^{th} cells embryo stage were transferred individually to wells of 96-well plates including 250 ml of diluted crude extract or standard water rearing media which were 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. Five concentrations (0.01%, 0.1%, 1%, 10% and 20%) of P. minima Linn. and a standard water control test were employed. All treatments consisted of 2 replicates. The well plates were covered with parafilm and placed in a temperature controlled room at 28^0C. 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) (Table1). The lowest observed effect concentrations (LOEC) and the no observed effect concentrations (NOEC) at 48 hpf were recorded.

Statistics: Non-parametric statistics were used to analyze the data. Hatching time and heart rate were analyzed with one-way ANOVA comparing groups. All other categorical parameters in the embryo toxicity test were analyzed using Fisher's exact test, in which each group was compared with the respective control group. The significance level was set at 0.95 ($p < 0.05$).

Table 1 Responses observation at 24, 48 and 144 hpf (Nagel, 2002; Braunbeck and Lammer 2006).

Responses	Observation endpoints		
	24 hpf	48 hpf	144 hpf
lethal	- Coagulation	- Coagulation	- Coagulation
Sublethal	- Movement	- Blood circulation	- Hatching time
		- Pigmentation	- Heart edema
		- Heart rate	- Abdominal edema
		- Heart edema	
		- Abdominal edema	
Malformation	- Fully development of somite, tail, eye and lens	- Fully development of somite, tail, eye and lens	
	- Tail detachment	- Tail detachment	

Results and Discussion

In this present study we used the zebrafish embryonic toxicity test method (FET) to investigate the toxic effect of the crude extract of *P. minima* Linn. leaf. The result is shown in Table 2 and Fig 1-3. We found that the lowest observed effect concentrations (LOEC) for the lethal effect and sublethal effect of *P. minima* Linn. on the zebrafish embryo were 20% and 10% respectively. This LOEC is higher than the LOEC of *Eclipta prostrate* Linn. and *Spilanthes acmella* (Linn.) Murr. in our previous test, which were 1% and 0.1% (Ponpornpisit et al., 2011). In this test, the no observed effect concentrations (NOEC) for the lethal effect and sublethal effect were 10% and 1% respectively. There was no observable malformation effect in any of the test concentrations. At the concentration of 10% and 20% of the crude extract there were two sublethal effects found in the embryos which were a slow heart rate and non-developed pigmentation (Fig 1 & 2). The same pigmentation effect in the fish embryo was also found in *Eclipta prostrate* Linn. and *Spilanthes acmella* (Linn.) Murr. FET test (Ponpornpisit et al., 2011). The absence of fish skin pigmentation could cause by either the blocking of melanin synthesis or the disruption of melanocyte development (Chen et al., 2012). According to the heart rate effect, 24 validation drugs, including doxorubicin, 5-fluorouracil, cyclophosphamide, mitoxantrone, terfenadine, clomipramine, thioridazine, gentamicin, tetracycline, amantadine, disopyramide, lidocaine, metoprolol, mexiletine, phenytoin, procainamide, propafenone, clozapine, erythromycin, quinidine, astemizole, amiodarone, verapamil, haloperidol, and clomipramine, have been studied in zebra fish *in vivo* cardiotoxicity assays. The results suggest that the drugs known to cause cardiotoxic adverse side effects in humans show similar effects in zebrafish (D'amico et al., 2011). Therefore, the slow heart rate effect resulting from *P. minima* Linn. crude extract should be of concern if anyone wants to use it in mammals. Although in our previous study we found 0.28 ng/ml progesterone in diethyl ether extraction (Suthikrai et al., 2010), this amount is less than melanocyte proliferation inhibiting level for the *in vitro* testing of human melanocyte (Wiedemann et al., 2009). A hormone can have different actions at different level administration. According to Snell (1962), administration of progesterone to guinea-pigs at 5 mg per day produced both stimulating and inhibiting effects on melanin pigmentation which differed in different skin regions and varied from one animal to another (Snell, 1962). The progesterone receptor was first found in human melanocyte cytoplasm and the nucleus by immunocytochemistry technique on melanocyte cell culture. In spite of the receptor being found, the influence on increasing and decreasing effect are unspecified (Sunbin et al., 2002). Plant origin flavonoids from licorice roots have shown a depigmentation effect on human melisma skin treatment (Situm et al, 2001). Soluble fractions of the extraction were orally given to male Wister rats at 1200 mg/kg without mortality appearing (Choudhary et al., 2007). Proliferation of melanocytes was induced by 17β-estradiol (0.1 and 1 nM) while progesterone (100 nM) reduced the proliferation rate of by 38% (Wiedemann et al, 2009). The reason that we did not reveal all *P. minima* Linn. chemical composite in this level was because of the discovery effect on embryos may not have resulted from only phytoprogesterone, therefore, further investigation of other chemical complexes that affect heart rate and pigmentation should be confirmed. According to this study, the application of 1% of *P. minima* Linn. extract can be applied to all animals stronger than zebra fish embryos without any effect. At 10% concentrations, the plant extract can be applied without lethal effect but clinical sign related to heart rate and the pigmentation effect should be monitored.

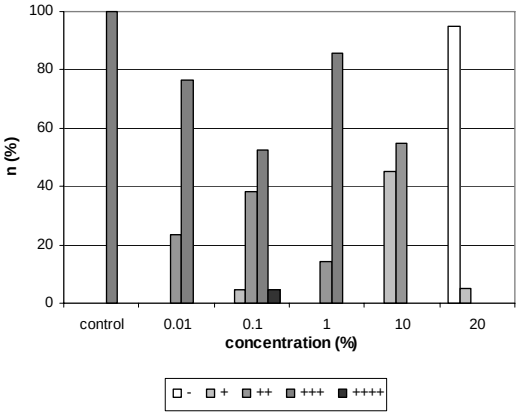

Figure 1 Comparison of the pigmentation of zebra fish embryo exposed to *P. minima* Linn. at 48 hpf. That the embryo pigment was totally inhibited at a concentration of 20% of the crude extract is statistically significant ($p < 0.01$). The embryo fully developed pigmentation in the unexposed group.

Table 2 Lowest observed effect concentrations (LOEC) and no observed effect concentrations (NOEC) at 144 hpf in zebra fish embryo test for *P. minima* Linn.

Endpoints	LOEC (%)	NOEC (%)
lethal	20	10
Sublethal	10	1
Malformation	-	20

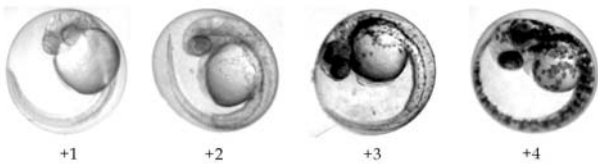

Figure 2 Pigmentation developing at different levels, the darkening embryo in the right picture represents +4 level of pigmentation developing score while the far left picture shows a +1 pigment score. Non-developed pigmentation or negative scores are not shown here.

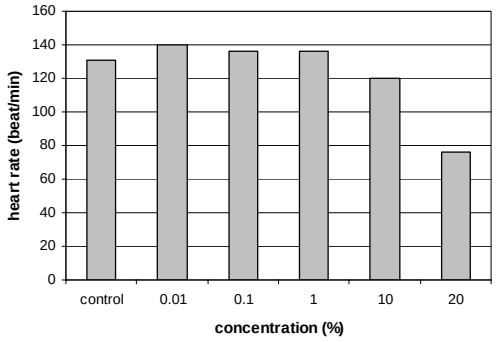

Figure 3 Heart rate of the zebrafish embryo test for *P. minima* Linn. was inhibited at 20 % of the crude extract. The heart rate in the 20% concentration was lower than other groups ($p < 0.01$).

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