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Bioassay-Guided Fractionation of *Emilia sonchifolia* Extract on the Induction of Ovarian Maturation in *Fenneropenaeus merguiensis*

(Pemeringkatan Bioasai Berpandu Ekstrak *Emilia sonchifolia* pada Aruhan Kematangan Ovari dalam *Fenneropenaeus merguiensis*)

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ABSTRACT

This study was carried out to identify the active compounds of Emilia sonchifolia *on induction of ovarian maturation in* Fenneropenaeus merguiensis. *The crude extracts from dichloromethane and acetone were investigated* in vitro. *The crude extract from acetone induced up-regulation of shrimp ovarian peritrophin (SOP) and translationally controlled tumor protein (TCTP) genes, which were highly expressed during early phase of ovarian development higher than dichloromethane extract. Furthermore, fraction 14 (F14) from acetone extract up-regulated both of the SOP and the TCTP genes to the greatest extent. Leading to* in vivo *study, the effect of ribosomal protein L10a (RPL10a), which acts as shrimp ovarian stimulator plus with F14 on shrimp ovarian maturation was investigated by injection. The best result was observed in the group that received RPL10a plus with F14 at 0.8 µg/g body weight of shrimp. The RPL10a plus F14 enhanced the shrimp ovaries from undeveloped stage to 57% of early developing stage within 15 days. Meanwhile, the control group remained 100% of the undeveloped stage. Hence, F14 seems to play a positive role in the induction of shrimp ovarian maturation. The component of F14 was identified using mass spectroscopy and presented as 1,2-benzenedicarboxylic acid; 2,4-di-tert-butyl phenol; 2,4,5-trimethoybenzylidene;palmitic acid; 1-heneicosyl formate; 1-heptadecanol; ethyl-4-ethoxybenzoate and stearic acid.*

Keywords: E. sonchifolia*; ovarian maturation; shrimp; SOP; TCTP*

ABSTRAK

Penyelidikan ini telah dijalankan untuk mengenal pasti sebatian aktif Emilia sonchifolia *pada induksi kematangan ovari dalam* Fenneropenaeus merguiensis*. Ekstrak mentah daripada diklorometana dan aseton dikaji secara* in vitro*. Ekstrak mentah daripada aseton meningkatkan aruhan peritofin ovari udang (SOP) dan secara translasi mengawal gen tumor protein (TCTP) yang dinyatakan pada fasa awal perkembangan ovari adalah lebih tinggi daripada ekstrak diklorometana. Selain itu, pecahan 14 (F14) daripada ekstrak aseton meningkatkan kedua-dua gen SOP dan TCTP ke tahap terbaik. Ini membawa kepada kajian* secara in vivo *dengan kesan ke atas protein ribosomal L10a (RPL10a), yang bertindak sebagai perangsang ovari udang tambahan F14 pada kematangan ovari udang yang dikaji melalui suntikan. Hasil terbaik yang diperhatikan dalam kumpulan yang menerima RPL10a dicampur dengan F14 pada 0.8 µg/g badan berat udang. RPL10a dan F14 ovari udang daripada peringkat mundur ke 57% peringkat awal pembangunan dalam masa 15 hari. Sementara itu, kumpulan kawalan kekal 100% pada peringkat mundur. Oleh yang demikian, F14 seperti memainkan peranan yang positif dalam aruhan kematangan ovari udang. Komponen F14 dikenal pasti menggunakan spektroskopi jisim sebagai asid 1,2-benzenadikarboksilik; 2,4-di-tert-butil fenol; 2,4,5-trimetoibenziliden; asid palmitik; format 1-heneicosil; 1-heptadekanol; etil-4-etoksibenzoat dan asid stearik.*

Kata kunci: E. sonchifolia*; kematangan ovari; SOP; TCTP; udang*

INTRODUCTION

Research on reproductive maturation in shrimp has been performed to increase the population of economically beneficial shrimp, especially the *Fenneropenaeus merguiensis* or banana shrimp which has diminished as a consequence of the increasing capture of wild shrimp for consumption. The control of the reproductive maturation in crustaceans has been studied mostly based on the endocrine system, which is related to gonad-stimulating hormone (Lee et al. 2011). For an example, to stimulate the maturation of prawn gonads is an eyestalk ablation, but this method causes high mortality and short-molt cycle duration in shrimp (Benzie 1998). The use of a diet with natural food, such as mollusk, squid and polychaete worms, is common and has been found to promote reproductive performance of shrimp broodstock (Meunpol et al. 2007), but it causes of pathogenic contamination to shrimp broodstocks. Therefore, the more effective methods to induce shrimp ovarian maturation are needed to investigate. Some studies reported that herbal products

are highly promising for utilization in the improvement of the reproductive performance and larval quality in shrimp aquaculture (Babu et al. 2008; Citarasu et al. 2002).

Emilia sonchifolia (L.) DC is a herbaceous plant and has been reported to contain alkaloids, tannins (Cheng & Röder 1986), flavonoids (Srinivasan & Subramanian 1980), sterols, palmitic acid and honey acid (Gao et al. 1993). The extract of *E. sonchifolia* have potent as antimicrobial activitiy (Chen et al. 2009), antioxidant, anti-inflammatory (Shylesh & Padikkala 1999), anti-tumor activities (Shylesh & Padikkala 1999; Yadava & Mamta 2012), anti-cancer (Shylesh et al. 2005) and antiviral activity (Maikaeo et al. 2015; Yadava & Mamta 2012). However, the compounds from this plant species have not been yet investigated in the area of controlling the reproductive maturation of animals. Thus, we aimed to study the biological effects of *E. sonchifolia* extract on shrimp ovarian maturation, in order to improve reproductive performance of broodstocks for a sustainable larval production of banana shrimp in aquaculture.

Materials and Methods

Plant material

E. sonchifolia was purchased from the local market in Hatyai, Songkhla, Thailand during March 2012. The leaves were washed with distilled water and air-dried. The dried leaves were soaked with dichloromethane or acetone solvent for 3 days, filtered and evaporated under vacuum to obtain the viscous crude extracts. A portion of crude extract was resuspended in 3 mL of 50% (v/v) methanol in dichloromethane and applied to a Sephadex LH-20 chromatography column. The bound material was eluted using 20 mL of 10% (v/v) methanol in dichloromethane. The eluate was collected as 14 fractions, aliquots werespotted onto TLC60 F_{254} silica gel sheets and then, were placed into either 50% or 10% (v/v) methanol in dichloromethane solvents. Following chromatography, spot patterns were visualized under ultraviolet (UV) light.

Shrimp ovarian explantsculture

Female wild-caught broodstock, *F. merguiensis* from Satun, Thailand were approximately 37.0±8.0 g. The shrimp with undeveloped ovaries were freshly dissected (approximately 1×0.5 cm) in M199 culture medium (1% v/v of fetal calf serum, 2% v/v of penicillin, 2% v/v of streptomycin and $NaHCO_3$, pH7.2).

Effect of crude extract on genes expression in shrimp ovarian explants (*in vitro*)

The three concentrations of dichloromethane or acetone crude extract were prepared at 0.5, 1.0 and 1.5 µg/mL inM199 culture medium. Crab saline (440 mM NaCl, 11 mM KCl, 13.3 mM $CaCl_2$, 26 mM $MgCl_2$, 26 mM Na_2SO_4, 10 mM HEPES, pH7.4) solution was used as control. The mixed culture media were filtered through 0.22 µm nitrocellulose membranes and were added to 48-well plates (500 µL per well) for triplicate measurements. Three pieces of ovarian explants were added to well plates and then incubated at 37°C and 5% CO_2 for 4 h. Ovarian explant were collected in Trizol reagent (Invitrogen, California, USA) for total RNA extraction. cDNA preparation, 4 µg of RNA was incubated with 200 ng random primers (Promega, Wisconsin, USA) at 70°C for 5 min and was cooled on ice for 5 min. Then, 1 mM dNTP mixture, 1 × reaction buffer and 10 U AMV reverse transcriptase (Promega, Wisconsin, USA) were added for a total volume of 25 µL and was incubated at 48°C for 2 h.

Gene expression analysis

The gene expression was determined by real-time PCR. The primer sequences used for targeting shrimp ovarian peritrophin (SOP), translationally controlled tumor protein (TCTP), vitellogenin (VTG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene amplification are shown in Table 1. The reactions for real-time PCR contained 100 ng of cDNA, 1 × FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) and 20 pmol each primer in a total volume of 25 µL. The PCR cycling program was initiated with a denaturing step at 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, annealing at 55°C (GAPDH and VTG) or 60°C (SOP and TCTP) for 30 s and extension at 72°C for 30 s. All genes were quantified by copy number from a standard curve, 10-fold serial dilutions from 1×10^7 to 1×10^3 copies were prepared for the calibration curve. The fluorescence detection employed an Mx3000P™ qPCR System (Agilent Technologies, California, USA).

Cytotoxicity of plant extracts on shrimp ovarian explants

In order to determine the cytotoxicity of plant extracts on shrimp ovarian explants, lactase dehydrogenase (LDH) enzyme that release from death cell was performed using an LDH detection kit (Roche, Mannheim, Germany). Briefly, culture medium after test was collected in microcentrifuge tubes and centrifuged (3,000×g, 5 min) to remove the cells. A 0.1 mL aliquot of supernatant was dispensed into 96-well microplate followed by 0.1 mL of LDH substrate for 30 min, then read at 490/630 nm using an automated microplate reader (BIO-TEK Instruments, Vermont, USA).

Induction of shrimp ovarian maturation by injection (*in vivo*)

The RPL10a protein was reported to induce shrimp ovarian development (Palasin et al. 2014). The application of PRL10a compared with the F14 combination was investigated to determine whether it could shift undeveloped oocytes to mature oocytes in shrimp. Female shrimps with undeveloped ovaries were randomly divided into 4 groups. Each group included 7 females and 2 males.

TABLE 1. Primer sequences used for quantifying the gene expression

Primer name	Primer sequences
Forward SOP	5′ CGGGATCCATGGCCACCACGACTAA 3′
Reverse SOP	5′ GCCTCGAGCCTGATGTTGTGTTTCTATA 3′
Forward TCTP	5′CCGCTAATTTCAAAAACTACG 3′
Forward TCTP	5′AAGGTGTTATGTCCAGGAAGT 3′
Forward VTG	5′ GTCAAGGAGAAGAACCACCGCC 3′
Reverse VTG	5′ CTGAAGGGATGCCGCTGATGG 3′
Forward GAPDH	5′ CAAGAAGGTCATCATCTCCGCT 3′
Reverse GAPDH	5′ TCCACGGTCTTCTGTGTGGC 3′

For the control group, female shrimps received buffer (350 mM NaCl, 50 mM NaH_2PO_4, 5 mM $MgCl_2$). The three challenged groups received only RPL10a protein (180 μg/g body weight (BW) of shrimp), RPL10a protein (180 μg/g) plus with F14 (0.8 μg/g BW. of shrimp) and RPL10a protein (180 μg/g) plus with F14 (8.0 μg/g BW. of shrimp). The female shrimps were injected 4 times at 0, 5, 7 and 10 days with 200 μL per shrimp in the third swimming leg. The shrimp were cultured for 15 days. Histology was used to determine the vitellogenic stage of shrimp ovarian maturation.

HISTOLOGY OF SHRIMP OVARY

In order to identify the stage of ovarian maturation after injection, ovarian tissue samples were collected and fixed in neutral, buffered formalin solution (10% of formalin, 33 mM NaH_2PO_4, 45 mM Na_2HPO_4) for 72 h. The fixed tissues were embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin. A microscope was used for observation of histological sections. The histological result was expressed as a percentage of the vitellogenic stage of shrimp ovarian maturation.

GAS CHROMATOGRAPHY -MASS SPECTROSCOPY (GC-MS) ANALYSIS

The phytochemical investigation of the F14 acetone extract was performed using GC-MS (GC; Hewlett Packard 5890, MS; Hewlett Packard 5972). The GC-MS system used an Innowax GC column (30 m long, 0.25 mm internal diameter, 0.25 μm film thickness) and a 260°C inlet temperature in the split-less mode for 0.8 min. The temperature program was 50°C for 5 min and an increase to 260°C at 5°C/min. The GC was connected directly to an electron ionization mass spectrometer with a range of 50-500 M/Z and the results were compared with the Wiley Spectral Library Database (9th edition, Wiley Registry™).

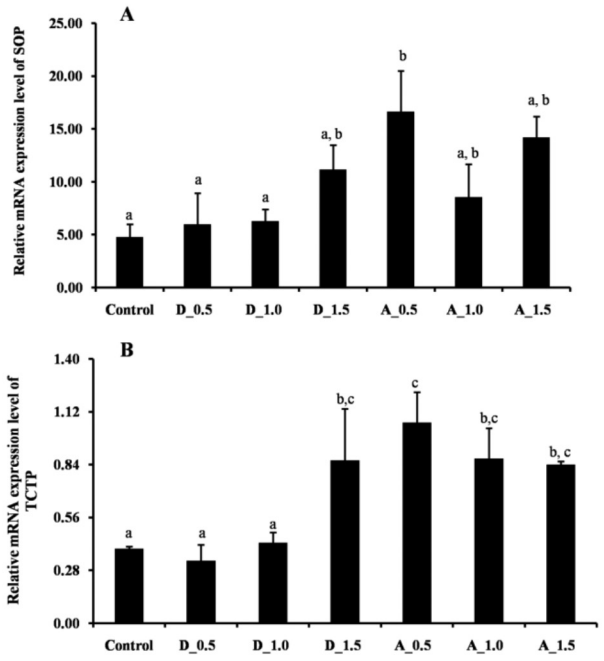
STATISTICAL ANALYSIS

The statistical significance of the data was determined using a one-way ANOVA and an independent paired t test with a 95% confidence level ($p \leq 0.05$) in the SPSS V16.0 software package.

RESULTS AND DISCUSSION

EFFECT OF AN ACTIVE FRACTION ON GENES EXPRESSION IN SHRIMP OVARIAN EXPLANTS (*IN VITRO*)

The bioactivities of dichloromethane crude extract and acetone crude extract were screened on induction of ovarian development in shrimp ovarian explants by gene expression of SOP, TCTP genes, which indicated the early vitellogenic stage and VTG gene, which indicated the late vitellogenic stage (Wonglapsuwan et al. 2010). Acetone crude extract can pronounced the up-regulation of SOP and TCTP gene expression in shrimp's ovarian explants higher than dichloromethane crude extract. In addition, acetone crude extract at 0.5 μg/mL was the most effective dose for induction of ovarian development by up-regulation of SOP and TCTP gene expression in ovarian explants (Figure 1).



D= Dichloromethane extract, A=Acetone extract (mean±SD, n=3, $P \leq 0.05$ ANOVA; different letters indicate significant differences)

FIGURE 1. Expression level of SOP (A) and TCTP (B) genes in shrimp ovarian tissue after exposure to extract fractions for 4 h

The SOP is a precursor protein of cortical rods and jelly layer during egg development, especially the early phase of vitellogenesis of banana shrimp (Wonglapsuwan et al. 2009). These evidences suggested that SOP is synthesized and accumulated at the beginning of oogenesis. The TCTP genes had been reported to have up-regulated expression during early phase of vitellogenesis of banana shrimp (Loongyai et al. 2007). The function of TCTP was proposed to be responsible for cytoskeleton organization for growth and development of oocytes (Lo et al. 2007). There were several publications demonstrated that the expression of SOP and TCTP genes related with oogenesis. The serotonin hormone (Makkapan et al. 2011) and RPS3a protein (Navakanitworakul et al. 2012) had a function *in vitro* to up-regulate the expression of SOP and TCTP genes were proposed to be related oogenesis in banana shrimp. These findings indicated that *E. sonchifolia* acetone extract can induce shrimp ovarian maturation via SOP and TCTP gene expressions.

Moreover, acetone crude extract contained an active compound was further fractionated using chromatography (Figure 2). Four fractions (F8, F10, F12, F14) with different TLC pattern were evaluated to induce gene expression in shrimp ovarian explants. The results showed that F14 significantly different induced the up-regulation of SOP and TCTP gene expression in ovarian explants compared to control ($p \leq 0.05$) (Figure 3).

For VTG gene expression, F14 incubated with early-developed ovarian explants significantly different up-regulated VTG gene compared to its control, while F14 incubated with undeveloped ovarian explants showed no significantly different up-regulated VTG gene compared to its control (Figure 4). The VTG gene is precursor of vitellin which is major yolk protein for shrimp eggs (Lee & Chang 1997). VTG is slightly increased their expression from early stage to strongly up-regulated during late stage of vitellogensis (Wonglapsuwan et al. 2010). This result indicated that F14 was more effective to induced oocyte development, where ovarian explants were in early-developed stage via VTG-induction.

The cytotoxic effects of plant extracts on shrimp ovarian explants were tested using the LDH assay. The release of LDH from the positive control (death cell) was significantly higher than both the treatments with plant extracts and control (Figure 5). Thus, the results indicated that the compound from this plant had no cytotoxicity to shrimp ovarian explants.

EFFECT OF AN ACTIVE FRACTION ON SHRIMP OVARIAN MATURATION (*IN VIVO*)

The positive results from *in vitro* experiment, F14 was injected in to female shrimp, subsequently ribosomal protein L10a (RPL10a) protein injection to monitor shrimp ovarian development by histology section of shrimp ovaries. The histology of undeveloped ovaries (Figure 6(a)) contained oogonia and follicle or nurse cells. For early-developed oocytes or stage I (Figure 6(b)) contained larger oogonia, called previtellogenic oocytes. The percentage of vitellogenic stage of shrimp ovaries after injection was calculated (Figure 6(c)). The results showed that the combination of RPL10a and a low dose of F14 (RPL10a+F14 at 0.8 µg/g BW) was an effective dose that increasing shrimp ovaries into 57% of early development stage within 15 days. Only RPL10a injection showed weak stimulation of ovarian development, was observed 29% of early development stage, while the shrimp ovaries in control group remained 100% of undeveloped stage. This result showed a similar to the RPL10a protein injection that can induce shrimp ovarian maturation into early developmental stage within 3-7 days (Palasin et al. 2014). However,

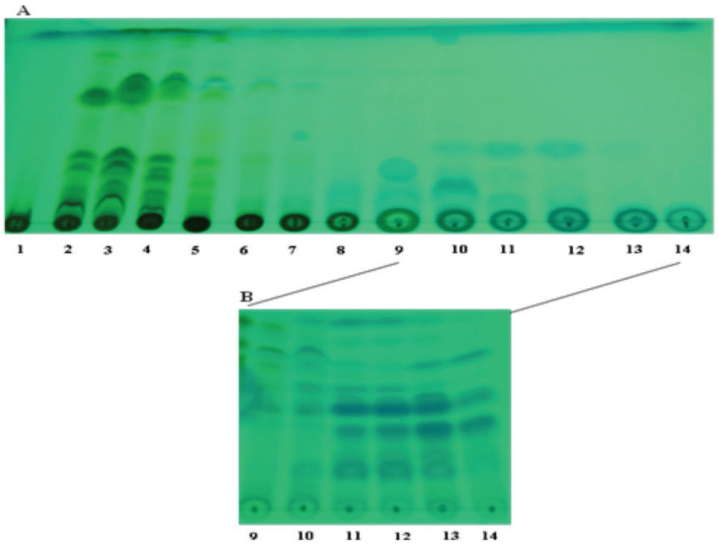
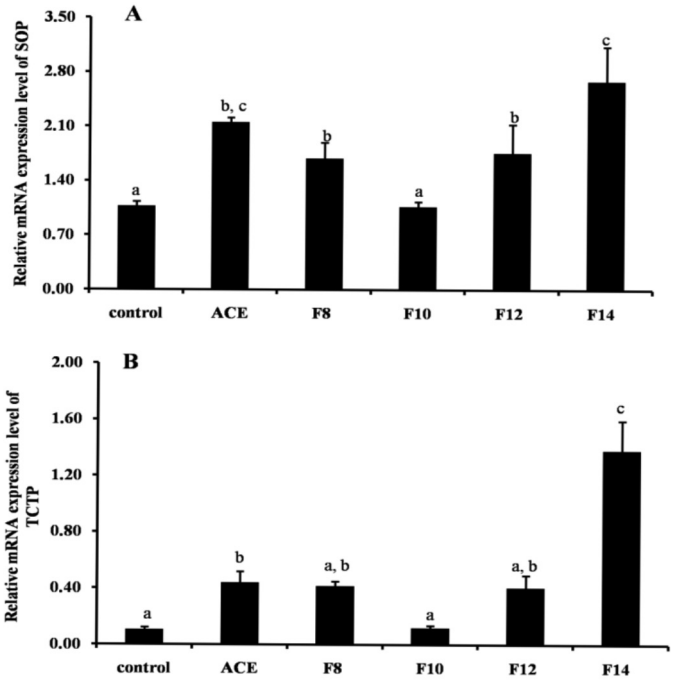
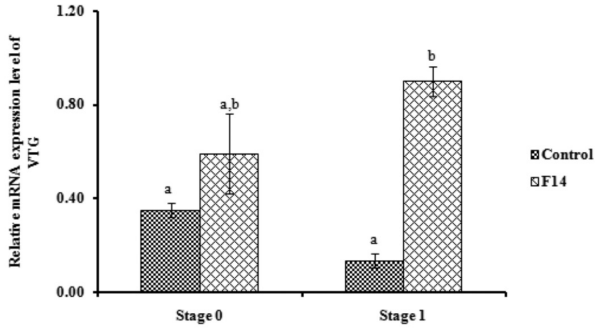


FIGURE 2. Characterization of *E. sonchifolia* acetone fractions by TLC, which were the firstly separated by mobile phase as 10% v/v of methanol in dichloromethane (A) and secondly using mobile phase was 50% v/v of methanol in dichloromethane (B)



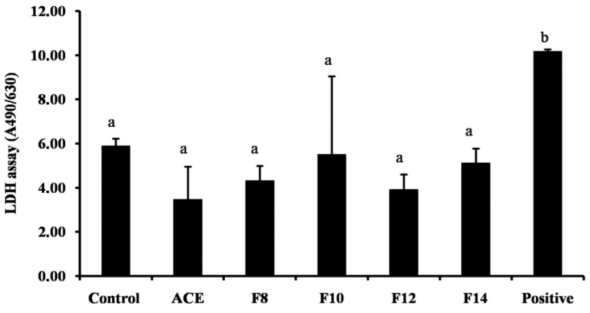
ACE=Acetone extract, F8=Fraction No.8, F10=Fraction No.10, F12=Fraction No.12, F14=Fraction No.14
(mean±SD, n=3, P≤0.05 ANOVA; different letters indicate significant differences)

FIGURE 3. Expression level of SOP (A) and TCTP (B) genes in shrimp ovarian explants after exposure to plant extracts for 4 h



(mean±SD, n=3, P≤0.05 independent paired t test; different letters indicate significant differences)

FIGURE 4. Expression level of VTG gene of shrimp ovarian explants after exposure to F14 for 4 h



(mean±SD and n=3, P≤0.05 ANOVA; different letters indicate significant differences)

FIGURE 5. Cytotoxicity of plant extracts on ovarian explants after 4 h incubation were detected by LDH assay

they were not able to induce shrimp ovarian maturation reached to mature stage. In this study, F14 was also effect on increasing the percentage of vitellogenic stage higher than only RPL10a injection. The results indicated that the bioactive molecules of F14 appear to play a role in the induction of shrimp's ovarian maturation.

Many plant species had been reported to use for shrimp ovarian induction (Devi 1995; Hilda 1992; Sambhu & Jayaprakas 2001). The female shrimp were fed with plant extracts show positive influence on the reproductive performance, biochemical parameters and larval quality (Babu et al. 2008). However, there are no information about chemical constituents in plant extracts which related to induce shrimp ovarian maturation. In this present studied, the preliminary phytochemicals in F14 from *E. sonchifolia* acetone extract was elucidated by GC-MS. The fingerprint of F14 showed eight main large peaks with different retention times (Figure 7). The mass spectra of each peak were identified and compared to spectral library. The result found that F14 contained as 1,2-benzenedicarboxylic acid, 2,4-di-tert-butyl phenol, 2,4,5-trimethoybenzylidene,palmitic acid, 1-heneicosyl formate, 1-heptadecanol, ethyl-4-ethoxybenzoate and stearic acid, as summarized in Table 2.

There are two compounds, palmitic acid and stearic acid, in F14 that were found at high levels in the

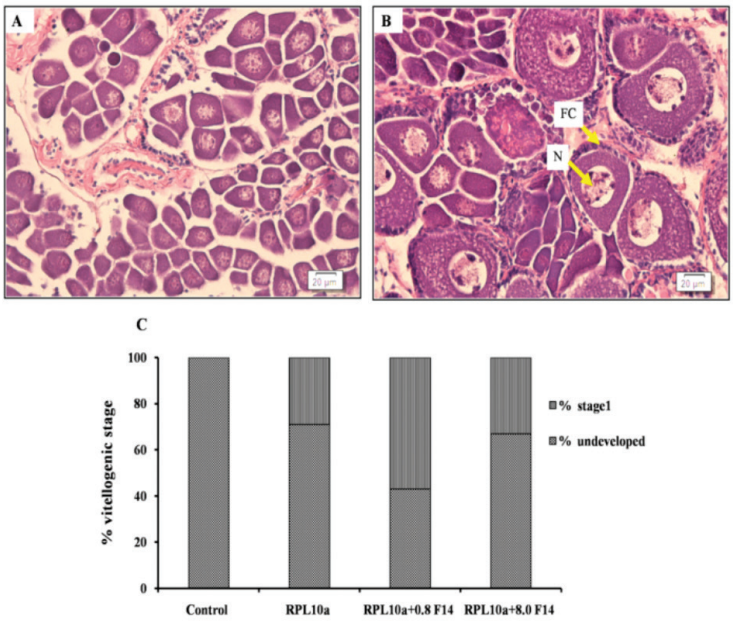


FIGURE 6. Histological section and vitellogenic stages of ovarian development of *F. merguiensis* after injection for 15 days (N≥7). Undeveloped ovary (A) and stage 1: Early developing oocytes (B) of shrimp ovaries, FC: follicle cells, N: nucleus. The results were expressed as percentage of vitellogenic stage (N≥7)

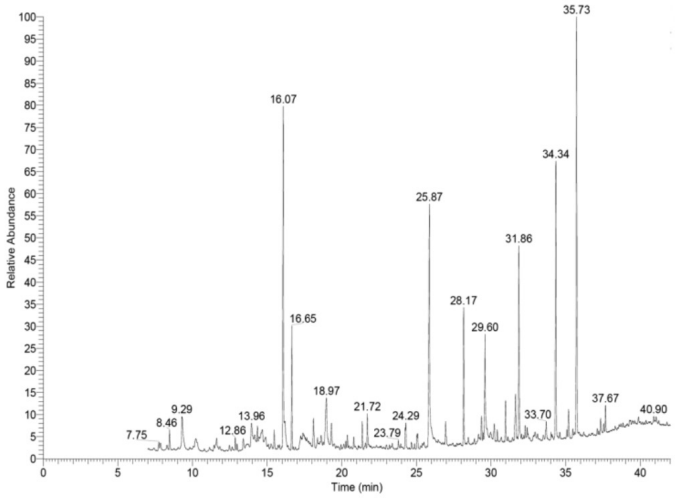


FIGURE 7. GC-MS chromatogram of F14 from *E. sonchifolia* extract

TABLE 2. Main components identified in F14 from the acetone extract by GC-MS

No.	Chemical compounds	Retention time	% Area
1	2,4-Di-tert-butyl phenol	16.07	13.61
2	Ethyl-4-ethoxybenzoate	16.65	4.38
3	Palmitic acid	25.87	10.32
4	1-Heptadecanol	28.17	4.51
5	Stearic acid	29.6	4.15
6	1-Heneicosyl formate	31.86	6.66
7	(2,4,5-Trimethoxybenzylidene)-Malononitrile	34.34	12.09
8	1,2-Benzenedicarboxylic acid	35.73	17.32

hepatopancreas, muscle and ovary during the reproductive development of shrimp (Maurício et al. 2012; Ouraji et al. 2011). These fatty acids are essential sources of energy in the maturing ovary (Xu et al. 1994), transported via the hemolymph, up-taken to the mitochondria by protein carriers and catabolized to acetyl CoA for the TCA cycle (Harrison 1990). Then, energy was used for the biosynthetic process of oogenesis and vitellogenesis in the ovaries (Teshima et al. 1998). These fatty acids in F14 may be due to stimulate the reproductive activity of shrimp.

Conclusion

F14 from acetone extract of *E. sonchifolia* leaves stimulated ovarian development in shrimp explants via SOP, TCTP and VTG genes induction. Moreover, F14 had been tested by injection to female broodstocks. The greatest effective of induction was observed in the shrimp received RPL10a plus F14. We can imply that F14 plays a role to stimulate the reproductive activity of shrimp. This finding was the first reports evaluating of *E. sonchifolia* extract on shrimp ovarian maturation. The results of present study may be useful for shrimp hatchery to regulate shrimp ovarian maturation.

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