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Synchronization of maturation in Silkworms, *Bombyx mori* L. (Lepidoptera: Bombycidae) using phytoecdysteroid from Purslane, *Portulaca oleracea* L.

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Abstract

Rearing of silkworms, *Bombyx mori* L. is an indispensable and significant activity of sericulture, the art and science of producing silk, the Queen of textiles. It is considered an arduous job and a critical concern is the unsynchronized maturation of silkworms on the onset of cocoon spinning requiring more time and manpower to complete mounting for cocoon production. This study determined the effect of phytoecdysteroid extracted from *Portulaca oleracea* L. on the synchronization of silkworm maturation to shorten the mounting time. High-performance liquid chromatography (HPLC) confirmed the presence of the phytoecdsyteroid, 20-hydroxyecdysone extracted from local plant, *P. oleracea*. The extract was sprayed with 50% and 100% concentrations (v/v) on mulberry leaves and administered to 5th instar silkworms different batches of silkworms at 116 hours, 92 hours, 76 hours, 52 hours and 28 hours before spinning. The time range and number of spinning silkworms during mounting process were recorded cumulatively. Results showed that both concentrations made approximately 50% synchronization of maturation in *B. mori* L. on the day of spinning at 6:30-7:59 AM and complete maturation at 8:00-9:59 AM. On the other hand, the negative control had approximately 50% synchronized maturation of silkworms between 10:00-11:59 AM and complete maturation at 1:00 PM. Phystoedysteroid from *P. oleracea* L. shortened the larval stage of silkworms and enhanced synchronization of maturation. Consequently, this study will help sericulture farmers consume lesser time and manpower of mounting matured silkworms.

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Introduction

Sericulture requires intensive labour, starting from the establishment of mulberry plantation intended for mulberry leaves production used primarily as food source of silkworms. Silkworm rearing is one of the components of sericulture which entails the raising of silkworms (*Bombyx mori* L.) for the production of cocoons which are processed into silk yarn and woven into fabric. It may also take about 5 to 7 rearings in tropical regions where mulberries thrive adequately throughout the year. Moreover, favorable conditions contribute to hefty income such as the variety of silkworms, the provision of nutritious mulberry leaves and equipping amicable management in rearing silkworm.

There are efforts being made generating new technologies that are cost effective, labour saving and eco-friendly. Numerous researches have been conducted mainly to increase production of quality silk that included mulberry propagation, silkworm breeding and rearing management, etc. Local researches also packaged and utilized technologies in the various aspects of sericulture in the farmer's site with the vital intention of producing good quality, bountiful cocoons enhancing productivity in sericulture.

In recent years, there were several attempts made to improve the quality and quantity of silk (Hiware, 2006 as cited by Srivastava & Upadhay, 2013) which include the enhancement of nutrients to the mulberry leaves, spraying with antibiotics, juvenile hormone, plant products and ecdysteroids. Incidentally, there are now remarkable and extensive investigations about ecdysteroids which are insect hormones involved in the development and the molting process (Gorelick-Feldman, 2009 as cited by Srivastava & Upadhay, 2012).

Edysteroids in plants are called phytoecdysteroids and are considered secondary metabolites, and produced in produced in small quantities. Secondary metabolites are not essential for growth and development of plant but these are required for the survival of plants in unfavorable environment. Secondary metabolites are important for flower color or volatile, flavor of food, attraction of pollinator, interaction with symbiotic microorganisms, and tolerance against pest and diseases (isoflavonoids and phenylpropanoid derivatives). These also take part in the mechanism of frost tolerance, nutrient storage, structural reinforcement, photo protective, UV–Vis absorption, signaling to mutualist, and in the adaptation of the plant under various environmental conditions and stress (Singer *et al.*, 2003; Verpoorte *et al.*, 2002 as cited by Sangwan, N. *et al.,* 2018). The two most common hormones found in insects are the ecdysteroid, 20E hydroxyecdysone (ecdysone) and the sesquiterpene, juvenile hormoneand and are usually accompanied by a number of other minor ecdysteroids (Naggar, *et al.*, 2017).

Ecdysteroid has been noticed to influence the reproductive potential of *Bombyx mori* (Pondeville *et al.*, 2008; Parlak *et al.*, 1992; Kawaguchi *et al.*, 1993 and Okuda *et al.*, 1993 as cited by Srivastava & Upadhay, 2012). Moreover, the application of very low doses has proven beneficial to improve the synchrony of development and yield of certain commercially important arthropods (Lafont and Dinan, 2009). The most extensively studied examples are silk moths (especially *Bombyx mori*), where protocols have been developed for the preparation of ecdysteroid containing extracts from native plants and application to the food plant (Morus spp.) at particular stages of development, which result in more synchronous cocoon formation and higher yields of silk per cocoon (Changrakala *et al.*, 1998 as cited by Naggar *et al.*, 2017), since there is unavailable related technology locally, this investigation was pursued to determine the effects of phytoecdysteroids in terms of synchronization of maturation of silkworms, *Bombyx mori* L. from purslane, *Portulaca oleracea* L.

Materials and methods

Plant Collection

The identified plant, *Portulaca oleceracea,* was properly described by De Guzman-Ladion (1985) and Kurian (2010) was collected along agronomic, horticultural fields and idle lands in the locality. The leaves and stems were properly washed with tap water and rinse with distilled water, and air-dried

until plant parts become well dried for grinding. After drying, the plant materials were mechanically ground into fine powder.

Preparation for Rotary Evaporation

For every 50 g of the powdered plant material, 150mL of the solvent (methanol) were used and soaked for 72 hours. After which, these were filtered using Whatman filter paper #4. The filtrate was subjected to extraction through rotary evaporation following the 20/40/60 rule (www.imlab.be), the condenser temperature (°C) was 20°, vapor 40°; bath temperature was 60°, and 74.51 torr for the pressure.

Chromatography

The presence of the phytoecdysteroids was confirmed through High Performance Liquid Chromatography (HPLC) outsourced from the De La Salle University, Central Instrumentation Facility, Chromatography Laboratory, Biñan, Laguna, Philippines. The specific standard, 20-Hydroxyecdysone ($C_{27}H_{44}O_7$) powder, ≥93% (HPLC) from Sigma Aldrich was purchased through a local distributor.

It made used of Agilent 1260 Infinity II HPLC using the detector G1314B Variable Wavelength Detector, InfinityLab Poroshell 120 EC-C18 4.6 x 150 mm with 4 micron particle size column, LCMS- grade acetonitrile (w/0.1% formic acid) and deionized water (w/0.1% formic acid) for the mobile phase, LCMS-grade methanol (MeOH) and deionized water for the reconstitution, 2mL microcentrifuge tubes, 1000μL micropipette, sonicator bath, microcentrifuge, and 2mL amber vials with silicon/PTFE septa screwcaps. The column oven temperature (°C) was at 40 during the test, and the Chemstation software was used for data acquisition and chromatogram processing.

Sample Preparation

Approximately 100mg of the slurry portion of the sample was weighed and transferred into 2mL microcentrifuge tubes. This was done in triplicates. After weighing, 1mL of 50:50 H2O:MeOH was added to the sample then capped immediately. This was then subjected to sonication at 25°C for 5 min then centrifuged at 14,000 rpm for 5 min.

The supernatant was obtained using the 1000μL micropipette then transferred into 2mL amber vials for analysis.

Standard Preparation

A 0.1mg of the standard was weighed and transferred into a 2mL microcentrifuge tube then 1mL of 50:50 H2O:MeOH was added, the microcentrifuge was capped, sonicated at 25°C for 5 min then centrifuged at 14,000 rpm for 5 min. This was transferred into a 2mL amber vial for analysis.

HPLC Conditions

The injection volume was 5μL; the Flow rate at 1.000mL/min, and the Mobile phase: Gradient of 0.1% Formic Acid in Water and 0.1% Formic Acid in Acetonitrile; the Max column pressure was at 400 bar; Column oven temperature was at 40°C; the variable wavelength detector was 242 nm; the run time was 23 min and equilibration time was 7 min.

Silkworm Rearing

The rearing of silkworm was conducted at the Sericulture Research and Development Institute, Bacnotan, La Union, Philippines. A week prior to the rearing activity, the rearing house and paraphernalia (rearing trays, nets and cocooning frames) were thoroughly cleaned and sanitized with 5% hypoclorite solution.

Newly-hatched bivoltine silkworm larvae were reared following the standard silkworm rearing management implemented by SRDI. The temperature and RH will be maintained at 26 – 28°C and 75 - 90% respectively. There were two-rearing trials conducted, covering the months of March-April and August – September.

The mulberry leaves enriched with phytoecdysteroid from the plant extract with 1:100 and 1:50 concentrations were administered *per os* to 5th instar silkworms at 116 hours, 92 hours, 76 hours, 52 hours and 28 hours before spinning.

The research design involved two-factor experiments in Complete Randomized Design (CRD) replicated three times in which factor S_5Dn corresponds the nth day of the 5th instar silkworms when the phytoecdysteroid-

enriched mulberry leaves were fed to the silkworms and C represented the concentrations.

Application of phytoecdysteroid (PE)

The methods of preparing the phytoecdysteroids and their applications by Rufaie *et al.* (2012) were adopted. The crude methanol plant extract was re-dissolved in methanol to prepare 10% stock solution (10g in 100mL). The solution was diluted further into 1:100 and 1:50 (v/v) concentrations of the extract in distilled water.

A single replicate contained 50 silkworm larvae fed 4 times a day at 6:00 and 10:00 in the morning; and 2:00 in the afternoon and 6:00 in the evening with mulberry leaves var. Alfonso was sprayed with PE concentration. The sets of replicates of the 5th instar silkworm larvae were prepared and fed with 150g mulberry leaves with 7.5mL of each of the concentration of 1:100 and 1:50 (v/v) at 116 hrs, 92 hrs, 76 hrs, 52 hrs and 28 hrs before spinning their cocoons.

The cumulative numbers of matured and mounted silkworms were gathered every after an hour at the 6th day of the 5th instar, which was the expected day of spinning their cocoons.

Results and discussion

Phytoecdysteroid (PE) in purslane, Portulaca oleracea L.

This investigation reveals the chromatograms yielded from HPLC analysis to determine the presence or absence of phytoecdysteroid specifically the 20-hydroxyecdysone in *P. oleracea*. Fig. 1 illustrates a sample blank chromatogram before undergoing analysis of the sample and standard.

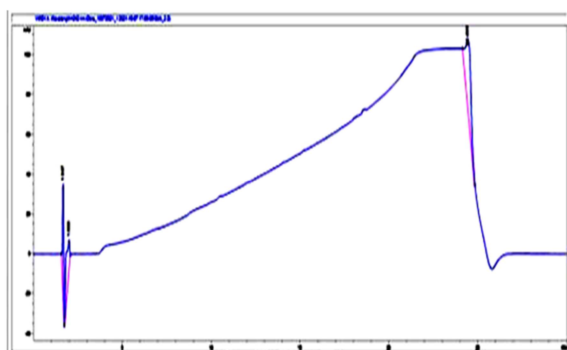

Fig. 1. Sample blank chromatogram.

The average retention time and peak area of the peak in the standard chromatograms A and B are shown in Figs 2 and 3. Fig. 2 shows the chromatograms for 100mg/L (ppm) of 20- hydroxyecdysone minus the sample blank chromatogram.

The chromatogram of the standard, 20-hydroxyecdysone which was analyzed after the sample is shown in Fig. 3.

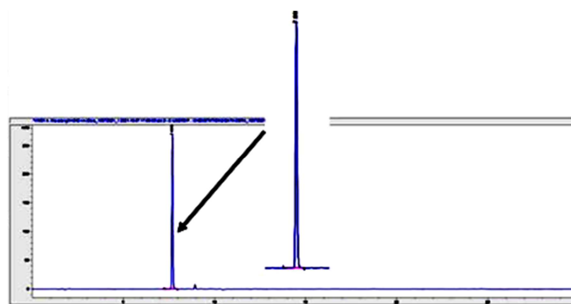

Fig. 2. Chromatogram of 100 ppm 20-hydroxyecdysone, analyzed prior to sample (Chromatogram A).

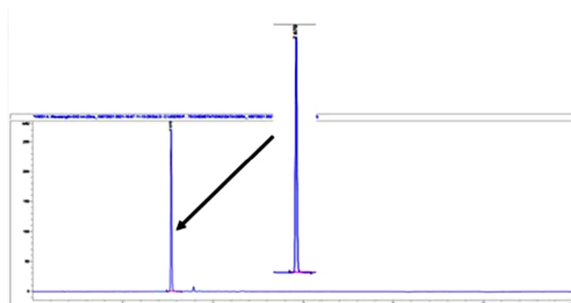

Fig. 3. Chromatogram of 100 ppm 20-hydroxyecdysone, analyzed after the sample (Chromatogram A).

The average retention time and peak are of the peak in the standard in chromatograms A and B (Figs 2 and 3) is shown in Table 1 by which the Retention time (min) is 7.670 ± 0.001 and the peak are is 1038.60 ± 0.57. There were 3 trials made, and the chromatograms for each of the trial for the sample are shown in Figs 4 to 6 showing the peaks, peak areas and retention time.

Table 1. Average retention time and peak are of the peak in the standard in chromatograms A and B (Figs 2 and 3).

Retention Time	Peak Area
7.670 ± 0.001	1038.60 ± 0.57

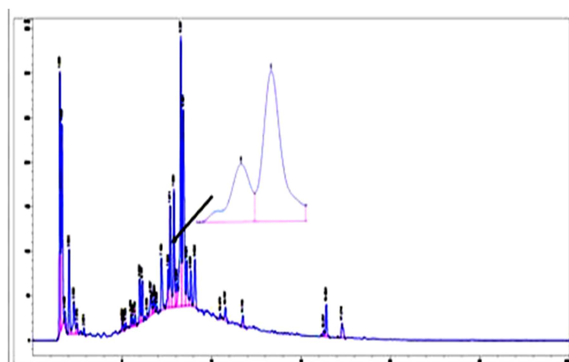

Fig. 4. Trial 1 chromatogram of the sample.

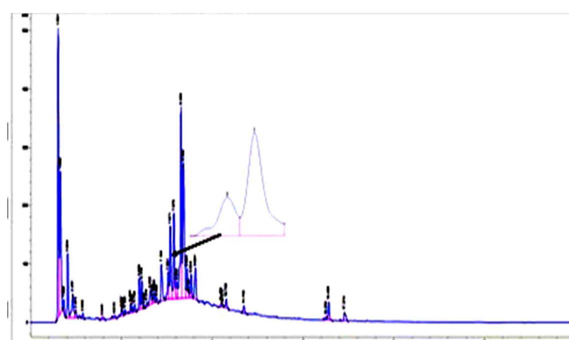

Fig. 5. Trial 2 chromatogram of the sample.

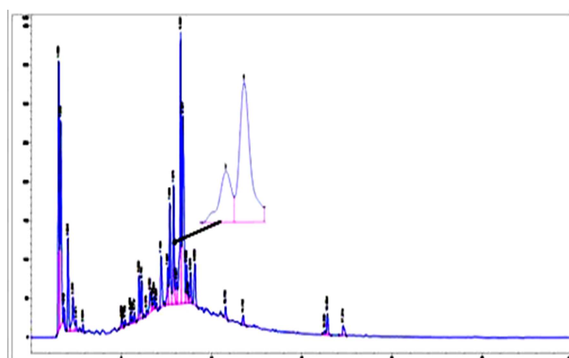

Fig. 6. Trial 3 chromatogram of the sample.

Table 2 shows the average retention time and peak area of the peak with the retention time close to the standard's retention time by a ± 0.120 deviation. Test for significant difference of the peak retention time versus the retention time of the standard's peak was determined using Welch's t-test. Retention time for Peak 1 was found to be significantly different from that of the standard, indicating possibility that the peak is not 20- hydroxyecdysone, but compounds belonging to the same class. The retention time of Peak 2 on the other hand, was found to be not significantly different from that of the standard, indicating that the peak is likely to be 20-hydroxyecdysone.

Table 2. Average retention time and peak area of the target compound in the sample.

Peak	Retention Time	Peak Area
1	7.560 ± 0.003***	235.13 ± 28.54
2	7.670 ± 0.002	593.63 ± 39.90

Welch's test for significant difference between retention time of sample peaks versus standard peak: *p < 0.05; **p < 0.01; ***p < 0.001.

Confidence interval = 95%; df = 2

Table 3 further illustrates the T-values of the peaks and retention time of the sample and the standard, 20-hydroxyecdysone. The sample contains 2 peaks close to the retention time of the target compound and the peaks with retention times are not significantly different from that of the standard were found, indicating presence of 20-hydroxyecdysone in the sample.

Table 3. T-values of the peaks and retention time of the sample and the standard, 20-hydroxyecdysone.

Peak no.	Retention time (min)	Two-tailed P value	Meanstd - Meansample (95% CI)	t value	Standard error of difference	Statistically different?
1	7.560 ± 0.003	0.0003	0.109 (0.101 to 0.117)	59.5455	0.002	Yes
2	7.670 ± 0.002	0.6985	-0.001 (-0.005 to 0.004)	0.4472	0.001	No

Given that the retention times of the peaks in the sample, Peak 1 of the sample is significantly different from the retention time of 20-hdroxyecdysone in Chromatograms A and B.

P. oleracea have been isolated including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals. The flavonoid contents of *P. olecacea* vary according to the parts of the plant on which the roots contain the highest levels followed by the stem and the leaf. There are seven different flavonoids present in the plant that includes kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin. Moreover, *P. olerac*ea contains monoterpenes such as portulosides A and B, diterpenes such as portulenes,

and β-amyrin type triterpenoids (Yan-Xi Zhou, 2015), however, it was not specified the phytoecdysteroid present in *P. oleracea*. The study of Daniel, M & Mammen, D. (2014) on the methanolic extract of *P. oleracea* yielded up to 0.532mg (in 100g of dry powder) of ecdysterone in addition to ƒsitosterol.

Synchronization of Maturation of Silkworms

The cumulative average number of matured 5th instar silkworm larvae that had been mounted at a specific time during the 6th day, which is the expected time of spinning their cocoons is presented in Table 4. It is shown that the Control group, silkworms that were not given the phytoecdysteroid had started to mature at 8:00 to 8:59 AM and reached complete maturation at 12:00 to 12:59 PM during the 1st trial and while the 2nd trial had reached until 1:00 PM.

Those silkworms provided with mulberry enriched with phytoecdysteroid had reached complete maturation between 8:00 to 8:59 AM on the 6th day. It presents that there is synchronized maturation of silkworms to be mounted for cocoon production. It signifies further that in the control groups of silkworms had longer larval period whereas those enriched with PE had shorter larval period regardless of concentration of the extracts, time of administration and trial when the study was conducted.

Table 4. Cumulative average number of matured 5th instar silkworm (*B. mori*) larvae for mounting at specified time during the 6th day.

Treatments (Age in Hours of 5th instar silkworms before spinning)	Trial	F (50)	Number of matured silkworms at: 6:30 - 7:59 AM	8:00 - 8:59 AM	9:00- 9:59 AM	10:00 - 10:59 AM	11:00- 11:59 AM	12:00- 12:59 PM	1:00 PM
Control	1	37	0	5	14	24	32	37	
	2	40	0	0	8	21	31	36	40
S5D1C1 (116 Hr)	1	44	22	44					
	2	41	20	41					
S5D1C2	1	42	17	42					
	2	39	17	39					
S5D2C1 (92 Hr)	1	41	20	41					
	2	40	19	40					
S5D2C2	1	42	23	42					
	2	41	18	41					
S5D3C1 (76 Hr)	1	43	21	43					
	2	42	18	42					
S5D3C2	1	42	20	42					
	2	40	18	40					
S5D4C1 (52 Hr)	1	43	20	43					
	2	42	19	42					
S5D4C2	1	44	20	44					
	2	43	19	43					
S5D5C1 (28 Hr)	1	42	23	42					
	2	43	19	43					
S5D5C2	1	43	21	43					
	2	44	18	44					

The above findings reinforced that of the statements described by Wong *et al*., 1979; Chow and Lu, 1980 as cited by Sashindran Nair, *et al*. (2005) that the use of PEs in sericulture during the latter stage of larval development of silkworms had the primary goal of accelerating larval development in the final larval instar and to synchronize the cocoon spinning process so that the larvae may be transported to the cocoon spinning device early and together, and the larvae can produce cocoons virtually simultaneously,

The synchronization of maturation of silkworms can save a lot of expert labor that would otherwise be necessary to pick up only the ripe worms and a large number of mulberry leaves in a timely manner.

According to Sehnal, 1989 as cited by Sashindran Nair, *et al*., 2005, the variation in larval and mounting time of silkworms is due to an exogenous ecdysteroid's physiological role in the insect development system. Ecdysone levels in feeding

larvae are always low, but they rise to a pupation-inducing peak before pupation. The pupation triggering peak of ecdysteroid content in silkworms is advanced by delivering an extra dosage of plant-based ecdysteroid at the key moment, changing the larval behavior as a result. The provision of mulberry leaves enriched with phytoecdysteroid from *P. oleracea* had also added to the needed amount of the ecdysone of the silkworm larvae for a synchronized maturation.

Since the maturation and spinning activities of silkworms are not uniform, it is a common practice by farmers that during silkworm rearing, silkworms are forced to be mounted in cocooning structures. Maturation and mounting may usually extend to 2- 3 days and even more during cooler seasons; this concern involves lot of time, labor and extra mulberry leaf and also ends up in higher production cost. It had been investigated that the administration of ecdysteroid, the problem could be avoided or minimized.

Fig. 7 illustrates the synchronization of maturation in silkworms of different treatments at specified time range. It can be deduced from the figure that the control groups took about six (6) hours while the treated groups, regardless of concentration and trial, had only two hours for complete maturation before mounting.

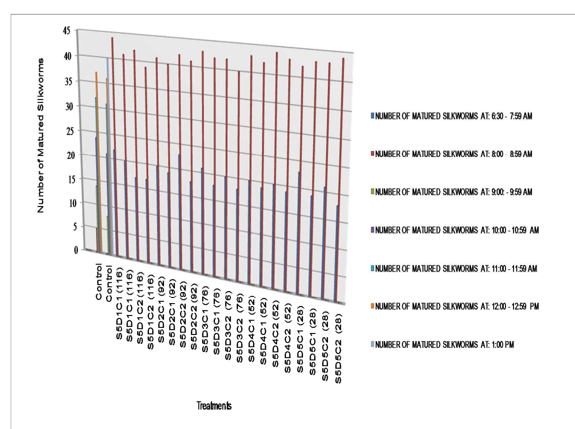

Fig. 7. Synchronization of maturation in silkworms of different treatments at specified time range.

It implied that *P. oleracea* contains PE; and if mulberry leaves were enriched with PE and administered to 5th instar larvae, it eventually enhances the synchronization of the maturation of silkworms.

Conclusion

High-performance liquid chromatography (HPLC) had established the presence of the phytoecdsyteroid, 20-hydroxyecdysone extracted from local plant, *P. olerace* L. It was found out that silkworms provided with mulberry leaves supplemented with the extract effected their synchronization of maturation regardless of concentration, trial and day or time of application of the 5th silkworm instar larvae compared to the negative control. Since it took approximately two (2) hours for all the treated silkworms to reach full maturation while six (6) hours for the control group, this manifested a shortened mounting time. Consequently, this study will help sericulture farmers consume lesser time and manpower of mounting matured silkworms.

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