Growth inhibition and feeding deterrence from leaf extracts of *Embelia ribes* Burm. f. on *Spodoptera litura* (F.) (Lepidoptera: Noctuidae)

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ABSTRACT: Six *Embelia ribes* Burm. f. leaf crude extracts prepared by fixed-bed (FB) and moving-bed (MB) methods with consequently extracted in hexane (H), dichloromethane (D) and methanol (M) were tested against *Spodoptera litura* (F.) larvae for growth and feeding deterrent properties. All crude extracts at 1,000 ppm, except crude methanol from leaves extracted by the FB method (FB/M), reduced growth of neonate *S. litura* larvae by 54 - 96% as compared to 73% of those larvae receiving neem seed extract. The best results were from dichloromethane extracts obtained from the MB method (MB/D) at 50 - 1,000 ppm where 59 - 98% reduced growth of neonate was recorded and the Effective Concentration value at 50 % (EC_{50}) was 20.9 ppm. While FB/D and neem seed extract at 50 - 1,000 ppm showed 51 - 84% inhibition rates,their EC_{50} were 24.9 and 63.6 ppm, respectively. Crude hexane and dichloromethane extracts, except FB/H, deterred feeding of 2nd instar *S. litura* larvae at the same level as the neem methanol extract as determined by a choice test method. The MB/D extracts induced 100% feeding deterrent index (FDI) while 91.25% was recorded for 1% neem methanol extract. A no-choice test experiment supported the choice test experiments. Leaf consumptions at 18.4 and 47.1% were recorded for 1% neem methanol extract and MB/D extracts, respectively.

Keywords: growth inhibition, feeding deterrent, crude extract, *Embelia ribes*, *Spodoptera litura*.

Introduction

Plant secondary metabolites have been the subject of thorough investigation for the past 30 years, in an effort to discover new sources of botanical insecticides. Screening of plant extracts for deleterious effects on insects is an approach used to search for new botanical insecticides (Isman, 1995). In addition, plant allelochemicals may act as pesticides, repellents, growth regulators, and antifeedants (Jermy, 1990).

Embelia ribes, commonly known as false pepper, is a woody shrub that belongs to the Myrsinaceae family. This plant species is sparsely distributed in the moist deciduous forests of the Western Ghats in India, Sri Lanka, Indo-Pakistan, Malaysia, South China, Vietnam, and Cambodia (Desai and Patil, 2000; Guha Bakshi et al., 2001, Dang et al., 2015). This plant also can be found in Chiang Mai, Ranong, Trang, and Nongkhai provinces in Thailand (Smitinand, 2001). The biological activities of *E. ribes* have been evaluated for antispermatogenic effects (Gupta et al., 1989) and urinary tract infections (Tripathi et al., 1992). Seeds are used as antibiotic, anthelmintic, and antituberculosis alternatives (Guha Bakshi et al., 2001). The leaves are astringent, demulcent, depurative, and useful for curing pruritus, sore throat, mouth ulcers, skin diseases, and leprosy (Sharma et al., 2002). The fruit contains a quinone derivative, embelin (3-undecyl 2,5 - dihydroxy, 1,4 - benzoquinone) and an alkaloid, christembine (Tyagi et al., 1978). Embelin has been reported to possess antifertility (Chong and Bachenheimer, 2000) and anti-implantation properties (Tatsuki et al., 1997). The berries have been reported to portray anthelmintic (Guru and Mishra, 1964), antibacterial (George

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and Pandalai, 1949), and antifertility activity in female rats (Radhakrishnan and Alam, 1975).

Only a paucity of information is available regarding bioactivity of *Embelia* leaf extracts against insects. However, embelin from *E. ribes* berries possesses grain protectant properties against several stored product insects such as *Callosobruchus chinensis* L, *Sitophilus oryzae* (L.), *Rhyzopertha dominica* (F.), *Corcyra cephalonica* (Sainton) and *Ephestia cautella* (Walker) (Chander and Ahmed, 1987, 1989), while petroleum and acetone extracts of *E. ribes* berries also show insecticidal activity against *Dactynotus carthamii* H.R.L. and *Tribolium castaneum* (Herbst) (Deshmukh and Borle, 1975; Chander and Ahmed, 1985). Chander and Ahmed (1987) found that embelin had non-contact toxicity to *S. oryzae* and *R. dominica* adults. The first report on insecticidal activity of *E. ribes* extract on lepidopteran larvae was produced by Desai and Patil (2000) who found that acetone extract of *E. ribes* berries showed antifeedant effects against 3rd instar larvae of *S. litura*. In addition, the first report on leaf crude extracts of *E. ribes* was performed by Noosidum et al. (2007), where crude dichloromethane extracts of fresh and dried leaves exhibited oral and contact toxicity on *S. litura* larvae under laboratory conditions.

This paper presents the growth retardant and antifeedant effects of *E. ribes* leaf crude extracts on *S. litura,* which is one of the major lepidopteran pests of vegetable crops and various economically important plants in Thailand.

Materials and Methods

Insects

Spodoptera litura larvae were collected from a cabbage field in Nonthaburi province, Thailand. The colony of *S. litura* were maintained on artificial diet (modified from Ahmed et al., 1998) under laboratory conditions at 25 ± 2 ºC; 75 ± 5 %RH with a photoperiod of 12 : 12 (L : D) for more than 2 generations. The adults were fed a 20% glucose solution through a cotton wick and eggs laid by the adults were transferred to a separate rearing container. The larvae were provided with an artificial diet daily.

Plant extracts

Leaves of *E. ribes* were collected from Doi Ang Khang, Chiang Mai province, Thailand. Voucher specimens in herbarium (#CHKU 00021) were deposited at the Bangkok Herbarium, Botanical Research Unit, Department of Agriculture, Bangkok, Thailand. Leaf samples were air dried at room temperature (28 - 32ºC) for 24 h and ground to a powder before extraction. The fixed-bed (FB) and moving-bed (MB) methods were employed to extract plant metabolites. Fixed-bed extraction (hot extraction) was done in a soxhlet extractor, where the samples were sequentially extracted with n-hexane (H), dichloromethane (D) and methanol (M) for 8 h. The moving-bed method (cold extraction) was performed by placing plant materials in a glass beaker containing n-hexane and stirred on a magnetic stirrer operating at 200 rpm for 8 h. The extracts were then vacuum-filtered through Whatman® No.1 filter paper and the residue was consequently extracted in dichloromethane and methanol by the same procedure. Solvents were removed on a rotary evaporator and the crude extracts were weighed and refrigerated at 10 ^{o}C for further experimentation. The six crude extracts used in this study were designated as FB/H, FB/D, FB/M, MB/H, MB/D and MB/M, where FB = fixed-bed extraction, MB = moving-bed extraction, H =

hexane, D = dichloromethane, and M = methanol, respectively.

Standard plant extracts

Neem seed extract served as a standard plant extract, which was prepared from neem seeds received from Doi Ang Khang, Chiang Mai province, Thailand. The seeds were air-dried, ground, and extracted with methanol by soaking over two days. The extract was then vacuum-filtered through Whatman® No.1 filter paper before concentrated in a rotary evaporator and held at 40 °C.

Chronic larval growth bioassay

Extracts were evaluated for growth inhibition effect on neonate *S. litura* larvae using a chronic bioassay. A 50 µl drop of crude extract at the concentrations of 10, 50, 100, 500 and 1,000 ppm was incorporated into artificial diets (0.001, 0.005, 0.01, 0.05 and 0.1 mg/g). Neem extract in the same concentrations with equal amounts of distilled water was used as a standard. Five neonate larvae were placed into a rearing tray containing treated or control diets and held at 25°C, 12 : 12 (L : D), for 10 days. Ten replications per treatment were completed. Control larvae were fed with regular diet and their weight was recorded after 10 days. The mean weight for each extract was expressed as a percentage of the control.

Feeding deterrent effect

Both leaf disc choice and no-choice tests were employed to determine the feeding deterrent effects of leaf crude extracts. In the leaf disc choice tests, the second instar larvae (6 days old) were starved for 4 h prior to each bioassay. Fresh castor bean leaf discs (8 mm diameter) were treated with 15µl of crude extracts (10% w/v) (0.75 mg/cm^2) that was distributed evenly on the leaf disc. The same amount of neem methanol extract was used as the standard, while solvent and water served as controls. Two discs, one treated and one control, were placed opposite of each other on moistened filter paper, in a Petri dish (9 cm diameter). One starved larva was placed in the center of each dish (30 replications/ treatment) for each treatment. A plastic lid with a 5.5 cm diameter hole covered with nylon mesh was placed on each Petri dish, and kept at 25°C for 6 h before leaf consumption was determined using a modified leaf area meter. The feeding deterrent index (FDI) for each treatment was calculated as (C - T)/(C + T) × 100 where C and T are the control and treated leaf areas consumed by the larva, respectively (Isman et al., 1990). Then, FDI index was transformed by LY = Log10 (Y + 1 - (min Y)) where LY is the transformed index and Y is the FDI index.

In the no-choice test, fresh castor bean leaf discs (8 mm diameter) were treated with 15µl crude extracts (10% w/v) (0.75 mg/cm^2) and distributed evenly on the leaf disc. The same amount of neem methanol extract was used as the standard while solvent and water served as the control. A single treated or control disc was introduced individually into the rearing cup, laid with moistened paper to maintain moisture. One 2nd instar larva, after being starved for 4 h, was placed in each rearing cup, where a treated or control leaf disc was provided as the food source (30 replications/treatment). All larvae were allowed to feed on each leaf disc for 6 h and the amount of leaf consumption was determined using a modified leaf area meter.

Statistical analysis

Analyses were performed on the data recorded from each set of experiments. Chronic larval growth was compared using one-way analysis for each separate factor of extraction methods and concentrations. Probit analysis was also used to calculate the effective concentration (EC_{50}) values and to calculate the respective 95% confidence intervals (Finney, 1978) with R version 3.0.1.) (R Core Development team, 2013). Feeding deterrent effect was compared with one-way analysis of variance and where appropriate, least significant difference test was used and significant differences were reported when $P < 0.05$. All data were arcsine square root transformed prior to analysis.

Results and Discussion

Chronic larval growth bioassay

All crude extracts inhibited larval growth of neonate *S. litura* in an inverse dose-dependent fashion when added to artificial diets at the concentrations of 010, 50, 100, 500 and 1,000 ppm (0.001, 0.005, 0.01, 0.05 and 0.1 mg/g, respectively) (**Table 1**). Neonates receiving 10 ppm extracts showed inhibition rates between 16.0 - 42.2% as compared to normal growth, while larvae fed with commercial neem extract exhibited only a 27.7% growth rate. In this experiment, increasing the dosage of leaf extracts from 10 to 1,000 ppm (100 fold) resulted in a drastic decrease in larval growth rate. At 1,000 ppm of extracts showed inhibition rates from 45.5 - 98.6% as compared to normal growth, while larvae fed with neem extract grew only 26.9% growth rate. In addition, a slight decrease in larval growth was detected in larvae receiving 50 ppm and higher concentrations. All crude extracts failed to reduce growth rate of neonate when 0.001% was applied. However, neonate larvae fed with 1,000 ppm of FB/D and MB/D inhibited 84.4 and 98.6% larval growth, respectively, while 1,000 ppm of commercial neem extract reduced the larval growth rate by 73.1% (**Table 1**).

Table 1 Growth inhibitory effect of *Embelia ribes* dried-leaf crude extract on neonate *Spodoptera litura*

Crude Extracts	Growth inhibition rate (% relative to control) [1/]				
	10 ppm	50 ppm	100 ppm	500 ppm	1,000 ppm
FB/H	16.0 aB	22.5 cB	43.3 cA	48.1 cA	54.0 cA
FB/D	42.2 aC	51.9 abBC	69.1 abAB	72.9 bA	84.4 bA
FB/M	25.8 aB	24.5 cB	29.7 cAB	35.0 cAB	45.5 cA
MB/H	27.0 aB	32.2 bcB	41.7 cAB	38.5 cB	59.1 cA
MB/D	39.8 aD	59.9 aC	78.9 aB	94.7 aA	98.6 aA
MB/M	35.4 aA	38.7 bcA	47.0 cA	50.4 cA	52.2 cA
neem	27.7 aB	54.9 abA	56.4 bcA	66.5 bA	73.1 bA

[1/] Means followed by the same letters are not significantly different at the 0.05% level as determined by LSD (α=0.05). Lowercase letters compares means in column, uppercase letters compare means in rows.

These result clearly indicated that the extraction method did not affect the growth inhibition properties of each solvent. All crude extracts at 0.1% intermediately inhibited the larval growth of *S. litura* except FB/D and MB/D. Crude dichloromethane extracts from dried leaves

tended to be more effective than crude hexane and methanol extracts, especially MB/D, which inhibited larval growth by 94.67 and 98.62% when applied at the concentrations of 500 and 1,000 ppm, respectively (EC_{50} = 20.9 ppm). Moreover, EC_{50} of FB/D extract and neem extract at 1,000 ppm were 24.9 and 63.3 ppm, respectively (**Table 2**).

Table 2 Effective concentration at 50% of *Embelia ribes* dried-leaf crude extract against neonate *Spodoptera litura*

Crude Extracts	EC_{50} (ppm) [1/]	95% Confidence interval (%)	Chi-square	Slope ± SE	Intercept ± SE
FB/H	547.9 c	188.1 - 13,225.1	5.6	0.6 ± 0.1	-1.5 ± 0.2
FB/D	24.9 ab	11.2 - 42.4	3.8	0.6 ± 0.1	-0.8 ± 0.2
FB/M	> 700 d	-	2.7	0.3 ± 0.1	-1.0 ± 0.2
MB/H	> 700 d	-	6.3	0.3 ± 0.1	-0.9 ± 0.2
MB/D	20.9 a	14.3 - 28.3	3.6	1.2 ± 0.1	-1.5 ± 0.2
MB/M	471.1 c	151.1 - 16,711.1	0.7	0.2 ± 0.1	-0.6 ± 0.2
neem	63.6 b	35.4 - 102.3	3.9	0.6 ± 0.1	-1.0 ± 0.2

[1/] Numbers followed by the same letters are not significantly different.

Wheeler et al. (2001) reported that 0.5% methanol twig extract of *Trichilia americana* (Sesse and Mocino) Pennington inhibited growth rates of neonate *S. litura* by 96%, which is slightly more effective than the dried and fresh leaf methanol extracts of *E. ribes,* where only 51 - 68 and 74 - 86% growth inhibition was recorded at the same concentration. Many plant extracts also induced growth retardation in neonate lepidopteran larvae. Janprasert et al. (1993) reported that rocaglamide in *Aglaia odorata* Lour extract has growth retardant properties against neonate *Peridroma saucia* Hübner (EC_{50} = 1.37 ppm). Akhtar and Isman (2004) revealed that 0.1% seed extract of *Melia volkensii* Guerke could inhibit growth rates of neonate *Trichoplusia ni* Hübner and *Pseudaletia unipuncta* Haworth with EC_{50} = 7.6 and 12.5 ppm, respectively. In addition, Leatemia and Isman (2004) found that seed extract of *Annona squamosa* L. reduced growth *S. litura* neonate where only 8-67% growth rate was reported (EC_{50} = 191.7 ppm), which is similar to our findings.

Feeding deterrent effect

The results from crude dichloromethane extracts significantly differed from crude methanol extracts in both tests (F = 8.3; df = 6, 69; P < 0.000). Both dichloromethane extracts and MB/H showed feeding deterrent effects on second instar *S. litura* larvae in choice test methods. The MB/D provided deterrent activity and appeared to be more effective (FDI = 100%) than those receiving neem methanol extracts (FDI = 91.3%). Crude MB/H deterred feeding at considerably lower rates, even though no significant difference was detected, when compared to dichloromethane extracts. In addition, all crude methanol extracts failed to show feeding deterrent properties on *S. litura* larvae (**Table 3**). However, all leaves extracted by the MB method exhibited stronger feeding deterrent effects compared to leaf extracts prepared by the FB method, except FB/D. Moreover, crude hexane extract prepared by FB using dried leaf samples could not inhibit larval growth (**Table 3**).

Table 3 Feeding deterrent index (FDI) of *Spodoptera litura* second instar larvae fed on castor bean leaf discs treated with 10% *Embelia ribes* leaf crude extract for 6 hours (choice test)

Crude extracts	Feeding deterrent index (FDI) [1/]	LY index [2/]
FB/H	-12.5 ±29.4	1.3 ± 0.4 b
FB/D	67.2 ± 28.2	2.2 ± 0.0 c
FB/M	-70.7 ± 31.4	0.5 ± 0.3 a
MB/H	73.5 ± 22.1	2.0 ± 0.3 c
MB/D	100.0 ± 0.0	2.3 ± 0.0 c
MB/M	-8.0 ± 31.6	1.1 ± 0.4 ab
1% neem	91.3 ± 8.8	2.3 ± 0.0 c

[1/] Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). [2/] FDI index was transformed by LY = Log10 (Y + 1 - (min Y)).

Janprasert et al. (1993) reported that crude acetone extract from *A. odorata* is able to deter feeding of *S. litura* larvae (LC_{50} = 4.8 ppm), while methanol extract of the same plant did not have any effect on larval feeding, which is similar to our findings. In contrast, Wheeler and Isman (2001) found that seed methanol extract of *T. americana* deterred feeding of 5th instar *S. litura* larvae (EC_{50} = 0.18 µg/cm^2). In addition, Mikolajczak et al. (1989) extracted secondary metabolites from 10 plant species using ethanol as the solvent, followed by hexane, and found that when 16-10,000 ppm were mixed with an artificial diet and fed to *Spodoptera frugiperda* (Smith) larvae, all crude ethanol extracts showed higher feeding deterrent activity than hexane extracts. Moreover, 100% larval mortality was observed in all ethanol extracts being tested, which contrasts with this finding, where no larval mortality was detected after being fed with 10% hexane dichloromethane or methanol leaf extract (750 µg/cm^2). Akhtar et al. (2003) stated that different larval stages had an influence on feeding deterrent activity. The 5th instar *T. ni* larvae showed a stronger response to seed extract of *M. volkensii* compared to 2nd and 3rd instar larvae, by reduced larval feeding (Akhtar et al. 2003). Wheeler and Isman (2001) stated that *T. americana* seed extract strongly deterred the feeding of 5th instar *S. litura* larvae with EC_{50} = 0.18 µg/cm^2. From this experiment, FB/D and MB/D were able to deter feeding of 2nd instar *S. litura* larvae, where EC_{50} values were equal to 320 and 250 µg/cm^2, which is considerably higher than those of *T. americana* extract.

Table 4 Leaf consumption (%) of *Spodoptera litura* second instar larvae fed on castor bean leaf discs treated with 10% *Embelia ribes* leaf crude extract for 6 hours (no-choice test)

Crude extracts	Leaf consumption rate (%)[1/]
FB/H	77.7 ± 3.7 d
FB/D	61.7 ± 9.2 c
FB/M	77.7 ± 8.4 d
MB/H	62.6 ± 9.7 c
MB/D	47.1 ± 12.7 b
MB/M	81.8 ± 3.5 d
1% neem	18.4 ± 2.2 a
ethanol	88.2 ± 4.9 d

[1/] Means±SE followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05).

Table 4 demonstrates that the no-choice test experiment agreed with choice tests ($F = 23.5$; df = 7, 479; $P < 0.000$). Within 6 hours, the controlled larvae consumed 88.2% of castor bean leaf discs treated with ethanol, while those fed on leaf discs painted with 1% neem crude extract avoided feeding and only 18.4% leaf consumption was recorded. The consumption rate of larvae fed on leaf discs painted with 10% methanol leaf crude extract ranged from 67-81%. Moreover, *S. litura* larvae on leaf discs containing 10% MB/D extracts consumed considerably less (47.1%) compared to the control. Hence, it can be concluded that only MB/D extracts contained feeding deterrent properties but to a lesser extent compared to 1% neem methanol extracts where only 18.4% of the leaf discs were consumed. Hummelbrunner and Isman (2001) found that thymol not only induced toxicity but deterred feeding of *S. litura* larvae while Xie et al. (1994) reported that *T. connaroides* extract acted as a growth inhibitor and deterred feeding of *S. litura* larvae as well. In addition, this results indicated that MB/D crude extract provided growth inhibitory effect to neonate *S. litura* larvae and also had feeding deterrent effect to 2nd instar of *S. litura* larvae. Similar to Chiu (1989) reported that one substance could affect insects in various aspects, such as toosendanin showed antifeedant, growth inhibitor, and stomach poison activities.

Conclusion and Suggestions

The results of this study were similar to other previous studies who used plant extracts to control various insect pests in a laboratory condition. The results clearly indicate that *E. ribes* leaf crude extracts, especially dichloromethane extract, could retard growth rates of neonate *S. litura* and deter feeding of 2nd instar larvae. However, the efficacy of these extracts when apply in field trial are not confirmed, further experiments need to be test to clarify their efficiency in a field condition.

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