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Full Length Research Paper

Acaricidal activity of *Hypocrella raciborskii* Zimm. (Hypocreales: Clavicipitaceae) crude extract and some pure compounds on *Tetranychus urticae* Koch (Acari: Tetranychidae)

Suradet Buttachon[1], Angsumarn Chandrapatya[1]*, Winanda Himaman[2] and Anake Kijjoa[3]

[1]Departmant of Entomology, Faculty of Agriculture, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.
[2]Forest and Plant Conservation Research Office, Department of National Parks, Wildlife and Plant Conservation, Bangkok 10900, Thailand.
[3]ICBAS - Instituto de Ciências Biomédicas de Abel Salazar - CIIMAR, Universidade do Porto, 4099-003, Porto, Portugal.

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The acaricidal activity of secondary metabolites from an entomopathogenic fungus, *Hypocrella raciborskii* Zimm. in controlling two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is explored in this study as a new agent for pest control. The crude extract was prepared in ethyl acetate. Secondary metabolites were elucidated using Nuclear Magnetic Resonance and High-Resolution Mass Spectrometry analysis. The major secondary metabolites of the crude extract were ergosterol, dustanin (15α, 22-dihydroxyhopane) and 3β-acetoxy-15α,22-dihydroxyhopane. The crude extract and pure compounds were used to determine their toxicity, repellency and oviposition deterrence against *T. urticae*, under the laboratory conditions. Crude extract of 3% w/v showed a strong contact toxicity (97% mortality, one day after treatment) and residual toxicity (80%, three days after treatment). Ergosterol induced 75% mite mortality in a residual toxicity bioassay. Females topically sprayed with 3% crude extract had their egg production reduced by 97%. Direct spraying of 3β-acetoxy-15α, 22-dihydroxyhopane and dustanin reduced egg production of *T. urticae* by 71 and 70%, respectively. Both crude extract and pure compounds had relatively slight repellency effect on *T. urticae*.

Key words: Fungal metabolites, bioassay, two-spotted spider mite.

INTRODUCTION

Fungi from genus *Hypocrella* are pathogens which belong to Division Ascomycota, Family Clavicipitaceae. They are known as entomopathogens infesting scale insects (Coccidae, Homoptera) and whiteflies (Aleyrodidae, Homoptera) and are quite common in tropical region, especially in the moist old-growth forests where the epizootics normally occur on their hosts (Hywel-Jones and Evans, 1993; Chaverri et al., 2008).

Several entomopathogenic fungi produce secondary metabolites, many of which are reported to exhibit insecticidal, phytotoxic, cytotoxic, antiviral, antifungal antitumor and antibacterial activities (Krasnoff et al., 1996; Pelaez, 2004; Jin-Ming, 2006). However, there is a paucity of information regarding secondary metabolites or toxins produced by *Hypocrella* due to the lack of available

*Corresponding author. E-mail: agramc@ku.ac.th. Tel: 662-942-8350. Fax: 662-561-4882.

cultures for studies (Isaka et al., 2003). Zhenjun and Lown (1990) isolated hypocrellins A and B which are pigments from *Hypocrella bambusae* (Berk. & Broome) Petch found in People's Republic of China. These substances showed potential to be used as photodynamic therapy of tumors. Consequently, hypocrellins A, B and C were identified as secondary metabolites from *Hypocrella* sp. collected in China where hypocrellins A exhibited photosensitive nematicidal activity and hypocrellins B and C showed only photodynamic activity (Jin-Ming, 2006). Watts et al. (2003) demonstrated that extracts of *Hypocrella raciborski* Zimm., *Hypocrella discoidea* (Berk. & Broome) Sacc. and *Hypocrella tamurai* (Henn.) had cytotoxic activity against sf9 insect cell lines with ID50 value ≤ 10 µg/ml. Isaka et al. (2009) investigated the secondary metabolites of *Hypocrella* and their *Ashersonia* anamorphs collected in Thailand and reported 3 hopane triterpenes which are zeorin (6α,22-dihydroxyhopane), dustanin (15α,22- dihydroxyhopane) and 3β-acetoxy-15α, 22- dihydroxyhopane) and postulated that hopane triterpenes may be suitable to be used as chemotaxonomic markers for *Hypocrella* and their *Ashersonia* anamorphs. Recently, Isaka et al. (2011) reported seven new lanostane-type triterpenes hypocrellols A-G and six new hopane-type triterpenes from *Hypocrella* sp. BCC14524 collected in Thailand where some biological activities were also mentioned such as growth inhibition against *Mycobacterium tuberculosis* and cytotoxic activity against human cancer cell lines. However, acaricidal activity of these triterpenes is not yet investigated.

Mortality of insects induced by ascomycetous fungi is thought to be caused by toxic substances released by the fungus (Roberts, 1981; Evans, 1988). Hence, fungal secondary metabolites may play an important role in fungi-insect interactions and such compounds may have insecticidal properties (Mongkolsamrit et al., 2010). In this study, the secondary metabolites of *H. raciborskii* Zimm. were elucidated and both crude extract and pure compounds were investigated for the acaricidal activity on the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), which is one of the important phytophagous mite infesting several economic plants in Thailand. The information gained from this study will be useful for further development of alternative pesticides for mite control.

MATERIALS AND METHODS

Fungal extract

Fungal material

The fungus used in this study was isolated from a scale insect collected in Kui Buri National Park, Prachuap Khiri Khan Province, Thailand in January 2009 and identified by Miss Winanda Himaman. Fungal sample with accession number IF 01781 has been deposited in the Forest Entomology and Microbiology Group, Forest and Wild Plant Conservation Research Office, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Extraction of the fungal metabolites

H. raciborskii was cultured on potato dextrose agar (PDA) plates and incubated at 27 to 28°C for 7 days. Ten fungal mats (1 cm in diameter) were taken from each plate and placed in an Erlenmeyer flask containing 100 ml of malt extract broth (MEB) before being incubated at 27 to 28°C in the rotary shaker set at 180 rpm for four days. These fungal biomasses were used as inoculums throughout the study. A plastic bag containing 200 g of cooking rice that was autoclaved at 121°C for 15 min was inoculated with 3 µl broth culture and incubated at 28°C for 30 days (total of 120 bags). Ten moldy rice bags were then emptied into 5 L Erlenmeyer flasks and 2500 ml of ethyl acetate were added to each flask. All flasks were kept for three days before the fungal materials and solvents were filtered through a Bucher funnel using #2 Whatman filter paper. The two aqueous layers were then separated using a separating funnel and the ethyl acetate solution was concentrated at reduced pressure yielding 52.34 g of crude extract. About 15 g of crude were set aside to be tested in an acaricidal activity bioassay. Then 37.34 g was dissolved in 500 ml of a 4:1 mixture of chloroform and ethyl acetate and then washed with water (3 × 500 ml). The organic layer was dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give 36.38 g of crude chloroform extract, which was applied on a column chromatography of silica gel (380 g) and the fractions were collected as follows: fractions 1-77 ($CHCl_3$-petrol, 1:1), fractions 78 to 151 ($CHCl_3$-petrol, 7:3), fractions 152-194 ($CHCl_3$-petrol, 9:1), fractions 195 to 209 ($CHCl_3$), fractions 210-245 ($CHCl_3$-Me_2CO, 9:1), fractions 246 to 269 ($CHCl_3$-Me_2CO, 7:3), fractions 270-294 ($CHCl_3$-Me_2CO, 3:7) and fraction 295 (MeOH). Fractions 42-52 (2.2 g) were combined and precipitated from a mixture of $CHCl_3$ and petrol to give a white solid of ergosterol (0.8 g). Fractions 109-118 (0.79 g) and fractions 119 to 153 (4.7 g) were combined and precipitated from a mixture of $CHCl_3$ and petrol to give white solid of dustanin (15α, 22- dihydroxyhopane**)** (5.5 g). Fractions 154-159 (1.12 g) and fractions 160-182 (2.78 g) were combined and precipitated from a mixture of MeOH and petrol to give white solid of 3β-acetoxy-15α, 22-dihydroxyhopane (2.78 g).

Stock colony of *Tetranychus urticae*

A colony of *T. urticae* was obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. All mites were cultured on mulberry leaves (*Morus alba* L.) placed on moistened cotton pads resting on sponges in plastic boxes. The mulberry leaves were examined every few days and replaced with fresh ones when over-crowding of mites and yellow leaves were observed. The colonies were maintained at room temperature (27 to 28°C, 60 to 65% RH). Adult females were placed on new leaves for 10 days and newly emerged adults were used for each experiment. All bioassays were conducted and carried out under the same environmental conditions as the culture.

Bioassays

The test materials included: the crude extract that was prepared at 0.1, 0.5, 1, 2 and 3% (w/v) using 95% ethanol as the solvent, 3 pure compounds (ergosterol, dustanin (15α,22-dihydroxyhopane) and 3β-acetoxy-15α,22-dihydroxyhopane) prepared at 0.5% (w/v), dicofol and neem extract (0.1%) were used as a positive and a standard, respectively.

Residual toxicity

An aliquot of 50 µl of each tested material was painted on the upper surface of each mulberry leaf disc (2 cm in diameter). Mulberry leaf discs painted with ethanol 95% were used as the control while

those painted with dicofol and neem extract served as a positive and a standard, respectively. Dried leaf discs were then placed on moistened cotton pads in glass Petri dishes (9 cm in diameter) and 20 *T. urticae* females (two to three days-old) were transferred from the culture with a small artist's brush into the center of each leaf disc (2 leaf discs/Petri dish, 5 Petri dishes/treatment). The rearing units were kept at 27 to 28°C, 60 to 65% RH where mite mortality, repellency (number of mites staying off the discs) and number of eggs were recorded daily for three consecutive days.

Contact toxicity

Twenty (20) *T. urticae* females (two to three day-old) from stock culture were placed on each mulberry leaf disc (2 cm in diameter) resting on moistened cotton pads in 9 cm Petri dishes (2 leaf discs/Petri dish, 5 Petri dishes/treatment). Each Petri dish was then sprayed separately with 500 µl of test material using a mini sprayer. The control dishes received 95% ethanol. The treated mites were kept at room temperature and observations were made daily for three consecutive days where mite mortality, repellency (numbers of females staying off the discs) and number of eggs were recorded.

Repellent and oviposition deterrent test

A choice bioassay was used in this experiment. Leaf discs were carefully punched off from each leaf to ensure that the midrib was in the middle of the leaf disc. A 25 µl aliquot of tested material was smeared on one-half of each leaf disc with a No. 1 camel hair brush. The other half of the leaf disc was treated in the same manner with its solvent. Control leaf disc was treated with 95% ethanol on one side and water on the other side. All treated leaf discs were left until completely dried before placing on a moistened cotton pad in plastic Petri dishes (2 leaf discs/Petri dish). Twenty 2 to 3 day-old *T. urticae* females from stock culture were placed on the midrib of each disc and the entire rearing units were kept at room temperature for 3 days. The number of mite that survived and number of eggs deposited on each side of the disc were recorded daily. Each treatment was replicated 5 times. Percentage of live mites and number of eggs on treated side were compared directly with 50% of the normal value that would be expected if there was no repellent effect. Oviposition deterent index (ODI) was calculated as (C-T)/(C+T)x100 where C is the number of eggs laid on the control disc and T is the number of eggs laid on the treated disc (Dimetry et al., 1993). Number of mites that fell in the water was also added as an additional measure of repellency.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) by SPSS version 15.0. Treatment means were compared by Tukey HDS test at $p = 0.05$.

RESULTS

Residual toxicity

The residual toxicity and repellency effect of *H. raciborskii* crude extract against *T. urticae* (TSSM) are shown in Table 1. At day 1 after exposure, the mites on the treated leaf disc with 3% crude extract showed the lowest survival rate of 67.5% due to 20.0% mortality and 12.5% repellency activities. After 2 and 3 days, the survival and mortality rates of mites on leaf discs treated with 0.5, 1, 2 and 3% crude extracts were significantly lower than the control. At the end of this experiment, only 10 to 34.5% of mites treated with 0.5 to 3% crude extracts remained alive which were significantly different from 98 to 99% of survival mites in the controls. Moreover, this survival rate was not different from those treated with dicofol (1.5%).

All pure compounds also showed a toxic effect on TSSM but at lower levels, except for mites treated with ergosterol where 75% mortality was noted at the end of experiment. At day 3 after application, the survival rate of all mites treated with pure compounds (25 to 52%) was significantly lower than control (98.4 to 99%). In addition, only the mites on leaf disc treated with ergosterol 0.5% was not significantly different with dicofol treatment. The result also indicated that the dustanin and 3β-acetoxy-15α,22-dihydroxyhopane had considerably very low activity as a repellent since only 0.5 to 2% of mites were repelled from the leaf discs at the end of the experiment. Neem extract also repelled the TSSM at the low rate (5.0%) and its toxicity was significantly lower than the crude extract and ergosterol. On the other hand, ergosterol and dicofol did not exhibit any repellency activity against TSSM.

The fecundity rates of TSSM fed on leaf discs treated with crude extract are presented in Table 2. At day 1 after treatment, mites treated with 0.5 to 3% crude extract, dicofol and neem extract laid considerably fewer eggs/day as compared to those treated with pure compounds and controls. As the time progressed, mites in all experiments deposited less number of eggs/day as compared to the controls, where 30 to 80% egg reduction was recorded for mites treated with crude extract. The maximum percentage of egg reduction (80.6%) was with the 3% crude extract which was close to the egg reduction induced by dicofol (91.4%). All pure compounds showed a percentage of egg reduction (23 to 43.8%) lower than those of the neem extract (66.2%).

Contact toxicity

The contact toxicity of crude extract and pure compounds on TSSM is presented in Table 3. At day 1 after exposure, 3% crude extract demonstrated a high contact toxicity to TSSM by only 3.5% of mites surviving which was significant as compared to the controls. In addition, no live mites were found on the leaf disc after day 2. Moreover, 2% crude extract, dustanin and 3β-acetoxy-15α, 22-dihydroxyhopane reduced number of mites by 55 to 70% at day 1 after exposure. Mite mortality rate increased with increasing time and only a 19.5% survival rate was found where mites were treated with 2% crude extract. However, this survival rate was not significantly different with 31.5, 39 and 41.2% induced by dustanin, 3β-acetoxy-15α, 22-dihydroxyhopane and 1% crude extract, respectively. The crude extract and their pure compounds showed no repellency activity, while mites treated with neem extract demonstrated only 6% repellency.

Table 1. Accumulated mortality and repellency rates of *T. urticae* females at 1, 2 and 3 days after being fed with mulberry leaf discs treated with different concentrations of crude extract of *H. raciborskii* and its secondary metabolites.

Treatment	Days after treatment								
	Day 1			Day 2			Day 3		
	Percentage of mite (mean ± S.E.) [1/]								
	Live	Dead	Repelled	Live	Dead	Repelled	Live	Dead	Repelled
Crude extract									
0.1%	95.0±2.85^{b}	2.5±2.50^{a}	2.5±1.37^{ab}	80.5±5.15^{de}	17.0±5.27^{ab}	2.5±1.37^{abc}	71.5±3.42^{ef}	26.0±4.23^{ab}	2.50±1.37^{abc}
0.5%	88.5±2.18^{b}	6.0±0.61^{a}	5.5±1.84^{ab}	57.5±5.00^{bcd}	35.5±4.57^{bcd}	7.0±1.66^{bcd}	34.5±5.09^{bcd}	58.5±4.08^{cde}	7.0±1.66^{bcd}
1%	88.5±2.03^{b}	4.5±2.29^{a}	7.0±2.00^{bc}	46.5±9.77^{b}	45.5±9.66^{bcd}	8.0±1.66^{cd}	17.0±7.33^{ab}	75.0±3.79^{def}	8.0±1.66^{cd}
2%	91.0±4.51^{b}	6.5±4.22^{a}	2.5±0.79^{ab}	42.3±6.85^{b}	54.2±6.66^{d}	3.5±0.61^{abc}	17.6±7.74^{abc}	78.9±7.68^{ef}	3.5±0.61^{abc}
3%	67.5±8.67^{a}	20.0±10.48^{a}	12.5±3.26^{c}	38.0±6.59^{b}	49.5±8.78^{cd}	12.5±3.26^{d}	10.5±3.66^{ab}	77.0±5.56^{ef}	12.5±3.26^{d}
Ergosterol 0.5%	98.0±1.22^{b}	2.0±1.22^{a}	0.0±0.00^{a}	48.5±11.58^{bc}	51.5±11.58^{d}	0.0±0.00^{a}	25.0±9.25^{abcd}	75.0±9.25^{def}	0.0±0.00^{a}
Dustanin 0.5%	97.0±1.46^{b}	2.5±1.12^{a}	0.5±0.50^{ab}	67.6±5.40^{bcd}	31.9±5.19^{bcd}	0.5±0.50^{ab}	46.5±7.97^{cde}	53.0±7.92^{bcde}	0.5±0.50^{ab}
3β-acetoxy-15α,22-dihydrohopane 0.5%	99.0±1.00^{b}	0.0±0.00^{a}	1.0±1.00^{ab}	79.5±7.13^{de}	18.5±7.65^{abc}	2.0±0.94^{abc}	52.0±11.97^{de}	46.0±12.11^{bcd}	2.0±0.94^{abc}
Dicofol	50.5±8.64^{a}	49.5±8.67^{b}	0.0±0.00^{a}	2.0±0.94^{a}	98.0±0.94^{e}	0.0±0.00^{a}	1.5±0.61^{a}	98.5±0.61^{f}	0.0±0.00^{a}
Neem	98.5±0.61^{b}	1.5±0.61^{a}	0.0±0.00^{a}	78.5±4.65^{cde}	18.0±5.44^{abc}	3.5±1.70^{abc}	66.5±3.76^{e}	28.5±4.91^{abc}	5.0±1.77^{abc}
Ethanol	100.0±0.00^{b}	0.0±0.00^{a}	0.0±0.00^{a}	100.0±0.00^{e}	0.0±0.00^{a}	0.0±0.00^{a}	98.5±0.62^{f}	1.5±-.62^{a}	0.0±0.00^{a}
Water	100.0±0.00^{b}	0.0±0.00^{a}	0.0±0.00^{a}	100.0±0.00^{e}	0.0±0.00^{a}	0.0±0.00^{a}	99.0±0.61^{f}	1.0±0.61^{a}	0.0±0.00^{a}

[1/] Means ± SE in column followed by the same letter are significantly different as determined by Tukey HSD test (α = 0.05). Accumulated mortality was calculated as mite died and stayed on leaf discs. Repellency rates were calculated as mite go down in the water.

The fecundity rates of mites treated with crude extract are demonstrated in Table 4. At 1, 2 and 3 days after treatment, mites treated with 0.1 to 3% crude extracts produced relatively similar number of eggs/day as compared to the pure compounds. Moreover, the daily egg production of mites treated with 0.5 to 3% crude extract (0.7 to 7.1 egg/day) did not significantly differ from the dicofol (1.1 egg/day) treatment. However, mites treated with 0.1% crude extract laid relatively same amount of eggs throughout the experiment when compared with those mites receiving neem extract. The number of eggs produced daily and the total egg production decreased with increasing concentrations and 3% crude extract could reduce the number of eggs by 97% which was similar to the dicofol treatment. The dustanin and 3β-acetoxy-15α, 22-dihydroxyhopane were more effective in reducing the egg production when compared to ergosterol since 70.9 to 71.4% egg reduction were recorded for 3β-acetoxy-15α, 22-dihydroxyhopane and dustanin and where only 43.5% reduction was noted for ergosterol. Neem extract also showed only 50.4% egg reduction.

Repellent and oviposition-deterrent bioassay

The repellency bioassay used leaf discs where half of the surface was treated with crude extracts

Table 2. Daily egg production and the total number of eggs produced by *T. urticae* females at 1, 2 and 3 days after being fed mulberry leaf discs treated with different concentrations of crude extract of *H. raciborskii* and its secondary metabolites.

Treatment	Egg/female/day (Mean ± SE) [1/]			Total egg production[1/]	Reduction (%)
	1 Day	2 Days	3 Days		
Crude extract					
0.1%	6.9±0.67^{cdef}	8.1±0.66^{efg}	8.2±1.14^{bcd}	426.9±33.77^{fg}	30.2
0.5%	4.5±0.41^{abc}	5.8±0.79^{cde}	6.4±1.08^{abcd}	261.3±24.06^{cd}	57.3
1%	5.1±0.69^{bcd}	5.4±0.36^{bcde}	8.4±0.60^{bcd}	272.4±20.99^{cde}	55.4
2%	5.5±1.02^{bcde}	5.4±0.34^{bcde}	6.6±1.20^{abcd}	267.3±24.64^{cd}	56.3
3%	2.2±0.54^{a}	3.2±0.56^{abc}	4.1±1.26^{ab}	112.6±19.60^{ab}	80.6
Ergosterol 0.5%	7.8±0.54^{ef}	5.3±0.98^{bcd}	7.8±1.34^{bcd}	343.3±49.26^{def}	43.8
Dustanin 0.5%	9.3±0.43^{f}	9.0±0.43^{fgh}	9.2±1.02^{cd}	470.7±5.57^{g}	23.0
3β-acetoxy-15α,22-dihydrohopane 0.5%	7.3±0.82^{def}	6.3±0.54^{def}	7.1±0.92^{abcd}	387.6±20.40^{efg}	36.6
Dicofol	2.1±0.32^{a}	0.7±0.12^{a}	2.8±1.24^{a}	50.1±7.84^{a}	91.4
Neem	3.5±0.94^{ab}	2.9±0.41^{ab}	4.8±0.34^{abc}	201.8±20.53^{bc}	66.2
Ethanol	9.6±0.16^{f}	11.4±0.68^{h}	10.1±0.25^{d}	611.3±13.36^{h}	
Water	8.9±0.56^{f}	10.2±0.40^{gh}	10.7±0.18^{d}	596.8±17.29^{h}	

[1/] Means ± SE in column followed by the same letters are not significantly different as determined by Tukey HSD test (α = 0.05). Total egg production was calculated as number of egg laid on leaf discs for three days. Reduction (%) was calculated as [(T×100)/C]-100 where C is number of total egg production on the control disc and T is the number of total eggs produced on the treated disc.

or pure compounds and the other half with their solvents (Table 5).

The results demonstrated that mites in the control (ethanol) were distributed somewhat equally on both half of the leaf discs until the end of experiment. At day 1 after application, the percentage of live mites on the treated side in 0.5, 1, 2 and 3% crude extracts were lower than the control side (<50%). The repellency activity of crude extract decreased as the time progressed and no repellency effect occurred at the end of experiment. In addition, dicofol and neem also demonstrated their repellency activity on day 1 after treatment where only 16 and 30% of live mites remained on the treated side. However, percentages of live mites on treated side in all experiments increased at day 2 after exposure.

On the other hand, the pure compounds did not show repellency activity. At the end of this experiment, the percentage of live mites on treated sides in all experiments was not significantly different from the control side. The egg percentages on treated side of leaf discs are also presented in Table 5. At days 1, 2 and 3 after exposure, percentages of eggs laid on the treated side were significantly lower than that of the control in 0.5, 1, 2 and 3% crude extracts. This phenomenon was also true for the mites treated with ergosterol, dicofol and neem extract.

The oviposition-deterrent index (ODI) of TSSM on leaf discs after half of disc were painted with crude extract and their pure compounds are shown in Table 6. At day 1 after exposure, mites treated with 1, 2 and 3% crude extract showed ODI >50%. The highest ODI (84%) was found when 3% crude extracts was applied. This result was similar to those mites receiving dicofol (80.3%) and neem (63%). Mites that received 0.5 to 2% crude extracts showed ODI (34.5 to 54.9%) close to ergosterol, dicofol and neem; 40.5, 80.5 and 63% were recorded, respectively.

The ODIs decreased as time progressed where only 3% crude extract and dicofol showed ODI >50% at the end of experiment.

DISCUSSION

The NMR and HRMS analysis of *H. raciborskii* crude extracts revealed that secondary metabolites consisted of ergosterol, dustanin (15α,22-dihydroxyhopane**)** and 3β-acetoxy-15α,22-dihydroxyhopane which was in agreement with Isaka et al. (2009) who reported that *Hypocrella* and their anamorph, *Aschersonia* species, produced three hopane type triterpenes, 6α,22-dihydroxyhopane (zeorin), 15α,22-dihydroxyhopane (dustanin) and 3β-acetoxy-15α,22-dihydroxyhopane. However, the zerorin could not be detected in our study. Boonphong et al. (2001) also found that dustanin and 3β-acetoxy-15α,22-dihydroxyhopane are the major components of *Aschersonia tubulata* Petch. which is an anamorph of *Hypocrella* fungus. The crude extract exhibited antimycobacterial activity with the minimum inhibitory concentration (MIC) of 12.5 µg/ml.

Table 3. Accumulated mortality and repellency rates of *T. urticae* females at 1, 2 and 3 days after being sprayed directly with different concentrations of crude extract of *H. raciborskii* and its secondary metabolites.

Treatment	Day after treatment								
	Day 1			Day 2			Day 3		
	% of mite (mean ± S.E.) [1/]								
	Live	Dead	Repelled	Live	Dead	Repelled	Live	Dead	Repelled
Crude extract									
0.1%	87.0±3.12^{ef}	13.0±3.12^{ab}	0.0±0.00^{a}	80.5±2.15^{de}	19.5±2.15^{ab}	0.0±0.00^{a}	79.0±2.18^{fg}	21.0±2.18^{a}	0.0±0.00^{a}
0.5%	68.5±8.16^{de}	31.5±8.16^{bc}	0.0±0.00^{a}	59.0±8.68^{cd}	41.0±8.68^{bc}	0.0±0.00^{a}	53.0±5.67^{cde}	47.0±5.67^{cd}	0.0±0.00^{a}
1%	60.0±2.85^{cd}	40.0±2.85^{cd}	0.0±0.00^{a}	43.5±3.02^{bc}	56.5±3.02^{cd}	0.0±0.00^{a}	41.2±2.91^{bcd}	58.8±2.91^{cde}	0.0±0.00^{a}
2%	30.0±12.12^{b}	70.0±5.42^{e}	0.0±0.00^{a}	24.5±7.00^{ab}	75.5±7.00^{de}	0.0±0.00^{a}	19.5±5.56^{ab}	80.5±5.56^{ef}	0.0±0.00^{a}
3%	3.5±1.87^{a}	96.5±1.87^{f}	0.0±0.00^{a}	0.0±0.00^{a}	100.0±0.00^{e}	0.0±0.00^{a}	0.0±0.00^{a}	100.0±0.00^{f}	0.0±0.00^{a}
Ergosterol 0.5%	60.5±10.23^{cd}	39.5±10.23^{cd}	0.0±0.00^{a}	57.0±9.73^{cd}	43.0±9.73^{bc}	0.0±0.00^{a}	55.0±9.12^{de}	45.0±9.12^{bc}	0.0±0.00^{a}
Dustanin 0.5%	35.5±7.96^{bc}	64.5±7.96^{de}	0.0±0.00^{a}	34.0±7.52^{bc}	66.0±7.53^{cd}	0.0±0.00^{a}	31.5±7.65^{bc}	68.5±7.65^{de}	0.0±0.00^{a}
3β-acetoxy-15α,22-dihydrohopane 0.5%	45.0±5.65^{bcd}	55.0±5.65^{cde}	0.0±0.00^{a}	41.5±4.72^{bc}	58.5±4.72^{cd}	0.0±0.00^{a}	39.0±4.72^{bcd}	61.0±4.72^{cde}	0.0±0.00^{a}
Dicofol	3.0±1.84^{a}	97.0±1.84^{f}	0.0±0.00^{a}	3.0±1.84^{a}	97.0±1.84^{e}	0.0±0.00^{a}	3.0±1.84^{a}	97.0±1.84^{f}	0.00±0.00^{a}
Neem	89.5±4.13^{ef}	7.5±2.74^{ab}	3.0±4.11^{b}	81.0±3.12^{de}	13.0±1.66^{a}	6.0±2.03^{b}	70.5±5.56^{ef}	23.5±4.85^{ab}	6.0±2.03^{b}
Ethanol	100.0±0.00^{f}	0.0±0.00^{a}	0.0±0.00^{a}	99.0±0.61^{e}	1.0±0.61^{a}	0.0±0.00^{a}	97.5±0.79^{g}	1.5±0.62^{a}	0.0±0.00^{a}
Water	100.0±0.00^{f}	0.0±0.00^{a}	0.0±0.00^{a}	100.0±0.00^{e}	0.0±0.00^{a}	0.0±0.00^{a}	98.0±0.50^{g}	2.0±0.50^{a}	0.0±0.00^{a}

[1/] Means ± SE in column followed by the same letter are significantly different as determined by Tukey HSD test (α = 0.05). Accumulated mortality was calculated as mite died and stayed on leaf discs. Repellency rates were calculated as mite go down in the water.

Both crude extract and pure compounds of *H. raciborskii* revealed the acaricidal activity on *T. urticae*, a serious pest of economic plants in Thailand. However, the effects of crude extract and pure compounds varied with the method employed, likely due to different modes of action in each experiment. Mites treated with 3% crude extract and dicofol using direct spray method showed only 3.5 and 3% survival rates at day 1 after exposure, while those that received neem extract and ethanol had 89.5 and 100% survival rates. In contrast, 3% crude extract applied as residual treatment was able to reduce only 32.5% of TSSM at day 1 after exposure. On the other hand, neem extract and dicofol could reduce up to 28.5 and 98.5% of mite when applied as residual treatment, respectively. This phenomenon is supported by Araújo et al. (2012) who stated that toxicity in the contact bioassays of *Piper aduncum* essential oil and its components against *T. urticae* most likely is attributed to the penetration of the toxins in the respiratory system while toxicity in the oral bioassays occurred in the digestion system. Therefore, the mites will be slowly affected than in contact bioassay.

The toxicity of *H. raciborskii* crude extract on *T. urticae* revealed that this crude extract might be useful as a biological mite control. This coincided with Watts et al. (2003) who stated that extracts of *H. raciborskii*, *H. discoidea* and *H. tamurae* could be suitable to develop further for application in biological insect control.

Interestingly, crude extract of *H. raciborskii* was only toxic to TSSM under residual and contact bioassays, but influenced fecundity by showing an egg reduction of 80 to 90%. This finding agreed with that of Rosas-Acevedo et al. (2003) who found that the exudates of *Hirsutella thompsonii* Mexican strain HTM120I reduced egg production of *T. urticae* by 100% 6 days after treatment by topical application. Additionally, we found that the components of the *H. raciborskii* crude extract, ergosterol, dustanin (15α,22-dihydroxyhopane**)** and 3β-acetoxy-15α,22-dihydroxyhopane were not as repellent as the crude extract. This finding suggested a synergy in the constituents of the crude extract which is responsible for the repellent action of *H. raciborskii* crude extract.

Table 4. Daily egg production and total number of eggs produced by *T. urticae* females at 1, 2 and 3 days after crude extract and its secondary metabolites of *H. raciborskii* were sprayed directly onto the mite.

Treatment	Egg/female/day (Mean ± SE)[1/]			Total egg production[1/]	Reduction (%)
	1 Day	2 Days	3 Days		
Crude extract					
0.10%	3.6±0.39^{bcd}	5.7±0.28^{cde}	6.9±0.38^{bc}	282.8±11.80^{cd}	56.1
0.50%	2.9±0.84^{abc}	2.9±0.61^{abc}	4.6±0.58^{b}	157.6±38.80^{abc}	75.5
1%	2.2±0.46^{abc}	4.0±0.41^{abc}	7.1±1.01^{bc}	150.3±5.08^{abc}	76.6
2%	1.5±0.39^{ab}	5.1±0.99^{bcde}	5.2±0.44^{b}	85.7±19.14^{ab}	86.7
3%	0.7±0.19^{a}	0.0±0.00^{a}	0.0±0.00^{a}	14.0±3.72^{a}	97.8
Ergosterol 0.5%	4.1±0.75^{cd}	12.6±1.76^{g}	11.6±0.70^{d}	363.6±67.04^{d}	43.5
Dustanin 0.5%	1.4±0.41^{ab}	9.1±2.30^{defg}	11.2±1.31^{d}	184.1±56.04^{bc}	71.4
3β-acetoxy-15α,22-dihydrohopane 0.5%	1.2±0.33^{ab}	7.1±0.50^{cdef}	11.8±0.83^{d}	187.0±23.78^{bc}	70.9
Dicofol	0.7±0.17^{a}	0.9±0.78^{ab}	1.1±0.65^{a}	16.1±5.21^{a}	97.3
Neem	5.9±0.68^{d}	4.8±0.71^{bcd}	5.5±0.67^{b}	293.4±18.35^{cd}	50.4
Ethanol	11.0±0.26^{e}	10.9±0.30^{fg}	10.7±0.38^{d}	643.9±16.15^{e}	
Water	10.1±0.65^{e}	9.8±0.35^{efg}	9.6±0.33^{cd}	591.5±24.98^{e}	

[1/] Means ± SE in column followed by the same letters are not significantly different as determined by Tukey HSD test (α = 0.05). Total egg production was calculated as number of egg laid on leaf discs for three days. Reduction (%) was calculated as [(T×100)/C]-100 where C is number of total egg produced on the control disc and T is the number of total eggs produced on the treated disc.

Table 5. Percentage of *T. urticae* females alive and laying eggs on treated leaf discs at days 1, 2 and 3 after painting with 25 µl of *H. raciborskii* crude extract and its secondary metabolites.

Treatment	Mean (%) of live mite[1/]			Mean (%) of egg laid[1/]		
	1 Day	2 Days	3 Days	1 Day	2 Days	3 Days
Crude extract						
0.1%	40 (15/38)	53 (18/34)	56 (18/32)	49 (121/244)	44 (236/537)**	49 (438/880)
0.5%	34 (12/37)*	48 (13/27)	50 (11/22)	33 (109/325)***	46 (241/524)	46 (377/818)*
1%	12 (4/34)***	28(8/29)*	42 (10/24)	23 (88/375)***	29 (211/726)***	30 (313/1027)***
2%	12 (4/33)***	27 (4/15)	40 (2/5)	24 (68/287)***	22 (118/537)***	34 (206/604)***
3%	12 (3/33)***	33(3/9)	50 (1/2)	8 (23/280)***	16 (74/463)***	17 (87/510)***
Ergosterol 0.5%	54 (19/35)	66 (19/29)	34 (10/29)	31 (70/226)***	49 (332/674)	52 (584/1120)
Dustanin 0.5%	73 (27/37)	70 (23/33)	48 (11/23)	84 (206/246)	73 (532/726)	67 (783/1163)
3β-acetoxy-15α,22-dihydrohopane 0.5%	87 (33/38)	62 (23/37)	60 (21/35)	88 (226/257)	71 (506/708)	68 (765/1119)
Dicofol	16 (5/31)***	30 (6/20)	54 (7/13)	10 (27/268)***	13 (64/504)***	16 (97/590)***
Neem	30 (11/37)*	57 (20/35)	50 (15/30)	19 (65/343) ***	26 (180/673)***	30 (235/794)***
Ethanol	47 (19/40)	50 (20/40)	54 (21/39)	47 (203/428)	49 (451/912)	41 (565/1390)***

***P**ercentage was significantly different from 50%: *P<0.05, **P<0.01, ***P<0.001. [1/]Means in column was calculated as mites on treated side/total mite where mean % of egg laid was calculated as eggs on treated side/total eggs.

Conclusion

The biological activity of fungal crude extract depended on various compounds present in the extract and our investigation showed that the crude extract of *H. raciborskii* caused high mortality, repellency and oviposition deterring activities but the pure compounds did not strongly show such activities. Ethanol also had no effect on mite mortality, repellency and oviposition deterring activity in our experiments. Hence, more than one compound could cause mortality, repellency and oviposition deterring action on TSSM. On the other hand, ergosterol revealed high mortality in experiments on residual toxicity and dustanin was the active compound when sprayed directly. The 3β-acetoxy-15α,22-dihydroxyhopane showed a reduction in egg production.

Table 6. Oviposition deterrent index (ODI) of *T. urticae* females on leaf discs at days 1, 2 and 3 on treated leaf discs with 25 µl of *H. raciborskii* crude extract and its secondary metabolites.

Treatment	Oviposition deterrent index (ODI)[1/]		
	1 Day	2 Days	3 Days
Crude extract			
0.1%	1.1^{b}	11.2^{bc}	-0.9^{ab}
0.5%	34.5^{bcd}	10.0^{bc}	9.0^{bc}
1%	54.9^{de}	45.3^{cde}	40.1^{cd}
2%	51.2^{cde}	32.0^{bcd}	30.6^{bc}
3%	84.0^{e}	67.5^{de}	65.5^{d}
Ergosterol 0.5%	40.5^{bcd}	4.0^{b}	-2.5^{ab}
Dustanin 0.5%	-67.1^{a}	-46.2^{a}	-34.4^{a}
3β-acetoxy-15α,22-dihydrohopane 0.5%	-76.0^{a}	-42.9^{a}	-37.4^{a}
Dicofol	80.3^{de}	74.5^{e}	68.4^{d}
Neem	63.0^{de}	46.3^{cde}	38.5^{cd}
Ethanol	5.0^{bc}	1.3^{b}	5.6^{bc}

[1/] Means in column followed by the same letters are not significantly different as determined by Tukey HSD test (α = 0.05). Oviposition deterent index (ODI) was calculated as (C-T)/(C+T)x100; where C is the number of eggs laid on the control disc and T is the number of eggs laid on the treated disc (Dimetry et al., 1993).

However, this study only screened the crude extract and its major constituents in the laboratory. Further studies are needed to evaluate the acaricidal effect of crude extract in greenhouse and field. It would also be valuable to test the efficacy of crude extract against small and soft-body insect pests.

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