SCREENING FOR MOSQUITO LARVICIDAL ACTIVITY OF THAI MUSHROOM EXTRACTS WITH SPECIAL REFERENCE TO *STECCHERINUM* SP AGAINST *AEDES AEGYPTI* (L.) (DIPTERA: CULICIDAE)

Damrongpan Thongwat[1,2], Urat Pimolsri[1,2] and Pradya Somboon[3]

[1]Department of Microbiology and Parasitology, Faculty of Medical Science; [2]Centre of Excellence in Fungal Research, Faculty of Medical Science, Naresuan University, Phitsanulok; [3]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Abstract. For over 50 years, biological control of mosquito larvae has depended mainly on plant extracts, fish, bacteria, protozoa, filamentous fungi, viruses or nematodes. In this study, we screened 143 mushroom samples from 44 confirmed species in Thailand for their mosquito larvicidal activity. One g% (w/v) aqueous extracts of dried powdered mushroom samples were tested against 3rd stage *Aedes aegypti* larvae. Four mushroom species, namely, *Thaeogyroporus porentosus*, *Xylaria nigripes*, *Chlorophyllum* sp and *Steccherinum* sp, and two unidentified species showed larvicidal mortality ranging from 10% - 70% and 18% - 90% for 24- and 48-hour exposure time, respectively. *Steccherinum* sp aqueous crude extract, after 48-hour exposure, did not show any larvicidal activity at 1,000 ppm, whereas that from ethanol, after 24-hour exposure, had 50% and 90% lethal concentration of 203 ppm and 412 ppm, respectively, with higher levels of mortality after 48-hour exposure. This is the first report of mosquito larvicidal properties of Thai mushroom extracts.

Keywords: *Aedes aegypti*, *Steccherinum* sp, crude extract, larvicide, mushroom

INTRODUCTION

Aedes aegypti (L.) (Diptera: Culicidae) is the main vector of dengue virus, the cause of dengue and dengue hemorrhagic fevers worldwide (Ratnam *et al*, 2013). In Thailand, to limit the disease outbreak, temephos (chemical larvicide) has been widely used for a long time against *Aedes* larvae (Chareonviriyaphap *et al*, 1999). Although temephos has very good efficacy, its contamination in the environment might be toxic to non-target organisms, including humans. Moreover, resistance to temephos has been reported (Jirakanjanakit *et al*, 2007; Sornpeng *et al*, 2009). Therefore, biological control offers an alternative safer method.

Biological agents can kill mosquito larvae in two ways: 1) they are parasites of the larvae, and 2) they are larvicidal substances. As regards the later approach, much attention has been paid to larvici-

Correspondence: Dr Damrongpan Thongwat, Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, 99 Moo 9, Tambon Tha Pho, Mueang, Phitsanulok 65000, Thailand.
Tel: +66 (0) 55 964676; Fax: +66 (0) 55 964770
E-mail: damrongpanth@nu.ac.th

dal substances from living organisms, especially from plants, mostly herbs. Microbial organisms, mostly fungi, produce toxic metabolites against mosquito larvae, *viz*, metabolites from *Aspergillus flavus, Chrysosporium lobatum, Penicillium* sp and *Podospora* sp show larvicidal activity against *Culex quinquefasciatus*, *Anopheles stephensi*, *Ae. aegypti* and *Anopheles gambiae* mosquitoes, respectively (Govindarajan *et al*, 2005; Geris *et al*, 2008; Mohanty and Prakash, 2009; Matasyoh *et al*, 2011).

In the Fungus Kingdom, mushrooms, mainly belonging to subdivision Basidiomycotina, consist of more than 14,000 species (Lindequist *et al*, 2005) and traditionally, mushrooms have been used for medical purposes because of their antibacterial (Bender *et al*, 2003; Lindequist *et al*, 2005), anti-fungal (Smania *et al*, 2003), anti-viral (Brandt and Piraino, 2000), anti-tumor (Zaidman *et al*, 2005; Zhang *et al*, 2007), anti-allergy (Min *et al*, 2001), anti-inflammatory (Kim *et al*, 2003; 2004), and anti-oxidant (Ajith and Janardhanan, 2007) properties. In addition, cordycepin (3′-deoxyadenosine) from fruiting body of *Cordyceps militaris* has been reported to kill 3rd instar of diamondback moth, *Plutella xylostella* (Kim *et al*, 2002). However, few studies have been conducted on mosquito larvicidal activity from mushrooms. A secondary metabolite, (oxiran-2-yl) methylpentanoate, from *Cyptotrama asprata* mushroom kills *Ae. aegypti* larvae with LC_{50} and LC_{90} values of 1.50 and 1.90 ppm, respectively (Njogu *et al*, 2009). More recently, Bucker *et al* (2013) reported larvicidal activity from *Pycnoporus sanguineus* mushroom against *Ae. aegypti* and *An. nuneztovari* with LC_{50} value of 156.8 and 87.2 ppm, respectively. Wild mushroom species, *viz*, *Amanita phalloides*, *Boletus* sp, *Lactarius densifolius*, *Lactarius gymnocarpoides*, *Russula cellulata* and *Russula kivuensis* demonstrate larvicidal activities against *Ae. aegypti*, *Culex quinquefasciatus* and *An. gambiae* (Chelela *et al*, 2014).

A variety of mushroom species commonly are found in tropical rain forests, but little is known concerning mosquito larvicide -producing mushrooms in Thailand. This study reports the screening of aqueous extracts of mushrooms in Thailand for mosquito larvicidal substance, and the evaluation mosquito larvicidal efficacy of aqueous, hexane and ethanol extracts of selected mushroom species against the *Ae. aegypti* mosquito.

MATERIALS AND METHODS

Mushroom collection and identification

One hundred and forty-three fresh mushroom samples were collected from Chiang Mai, Krabi, Lampang, Nakhon Ratchasima, Nakhon Sawan, Pathum Thani, Phichit, Phitsanulok, Phuket, Prachuap Khiri Khan, Sukhothai, Surat Thani and Tak Provinces, Thailand and transferred to the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, where voucher specimens were deposited. Mushroom samples were identified macroscopically and microscopically following mushroom taxonomic keys (Largent and Thiers, 1977; Largent *et al*, 1977; Stuntz, 1977; Watling, 1977; Largent, 1986; Largent and Baroni, 1988). Samples were dried at 45°C for 24 hours, then ground into powder using a blender (Single Speed Blender 800G; MRC, Holon, Israel) at 22,000 rpm and stored at 4°C until used.

Mosquito rearing

Laboratory strain *Ae. aegypti* originally collected from Mueang District, Phitsanulok Province, Thailand was reared as previously described (Thongwat

et al, 2014). In brief, larvae were reared in tap water, at 25 ± 2°C with 10:14 light:dark photoperiod, and fed with powdered dog biscuits (Adult Complete Nutrition, PEDIGREE®; Mars Petcare, Franklin, TN). After pupation, pupae were transferred to a mosquito cage (30x30x30 cm) and emerging adults were provided with a 5% sugar solution containing 5% multivitamin syrup (SEVEN SEAS®; OLIC, Feltham, Middlesex, UK). Five- to 7-day-old females were given a blood meal using an artificial membrane feeding method (Rutledge *et al*, 1964). Gravid females were allowed to lay eggs on a wet filter paper (Whatman Nº 1) and eggs were air-dried for 3 days, then kept in a humidity controlled glass jar until used.

Larvicidal activity screening

Two grams of each mushroom powder were suspended in 200 ml of distilled water and agitated at 180 rpm for 24 hours on a rotary shaker (Innova™ 2300; NEW BRUNSWICK SCIENTIFIC, Edison, NJ) at room temperature. Then each suspension was filtered through a fine net cloth and added to 25 3rd instar *Ae. aegypti* larvae. Mortality rate was examined after 24- and 48-hour exposures with no feeding. Experiments were performed in four replicate. Controls contained distilled water.

Preparation of *Steccherinum* mushroom crude extracts

Ten grams of powdered *Steccherinum* sp, sample CKW03, were suspended in 100 ml of hexane and then continuously stirred at 180 rpm for 24 hours on a rotary shaker and filtered as described above. The residue was then extracted with ethanol followed by distilled water as described above. The hexane and ethanol extracts were dried in a rotary the evaporator (BÜCHI Rotavapor® R-205 equipped with BÜCHI Vac® V-500; BÜCHI, Flawil, Switzerland), while water extract was dried by evaporation and lyophilization (Lyotrap LF/LYO/01/1; LTE SCIENTIFIC, Oldham, UK).

Larvicidal bioassay

Dose-mortality bioassay against *Ae. aegypti* larvae was conducted following protocols of WHO (2005). In brief, 1 g% (w/v) stock solutions in dimethylsulfoxide (DMSO) of the crude ethanol and hexane extracts or in water for the aqueous extract were serially diluted in water and 200 ml aliquots were added to 25 healthy 3rd instar *Ae. aegypti* larvae. After 24 and 48 hours, mortality rates were recorded. Controls contained either 1% (v/v) DMSO or distilled water alone.

Data analysis

The 50 (LC_{50}) and 90% (LC_{90}) lethal concentrations and were determined using Probit analysis (Finney, 1971) with LdP Line® software (Plant Protection Research Institute, Cairo, Egypt). The 95% confidence intervals (CI) of upper and lower fiducial limits were also calculated. Statistical significance is accepted when a p-value is < 0.05.

RESULTS

Of 143 mushroom samples, 136 were identified into 46 genera with at least 44 confirmed species. The remaining 7 samples were unidentifiable because of limitation in quantity and incomplete morphology of the specimens. Larvicidal activity of all mushroom aqueous extracts [(1g% (w/v)] showed that 4 identified [*Chlorophyllum* sp (NU01), *Steccherinum* sp (CKW03), *Thaeogyroporus porentosus* (PHK27), and *Xylaria nigripes* (PW03)] and 2 unidentified (CKW05 and GSW04) specimens displayed larvicidal efficacy ranging from 10% - 70% and 18% - 90% lar-

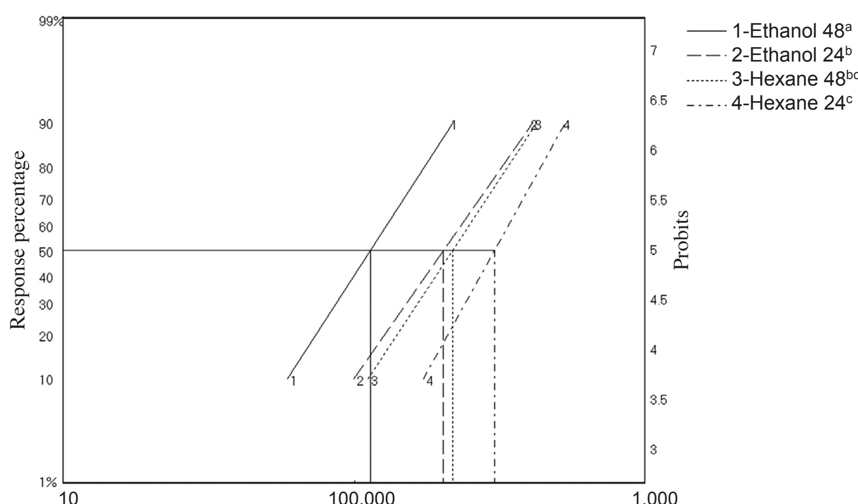

Fig 1–Graph showing LC_{50} values of ethanol and hexane crude extracts of *Steccherinum* sp mushroom against *Ae. aegypti* 3rd instar stage (n = 25) at 24- and 48-hour exposure. X-axis denotes extract concentration in ppm. Statistically significant differences are indicated by different letters (upper right).

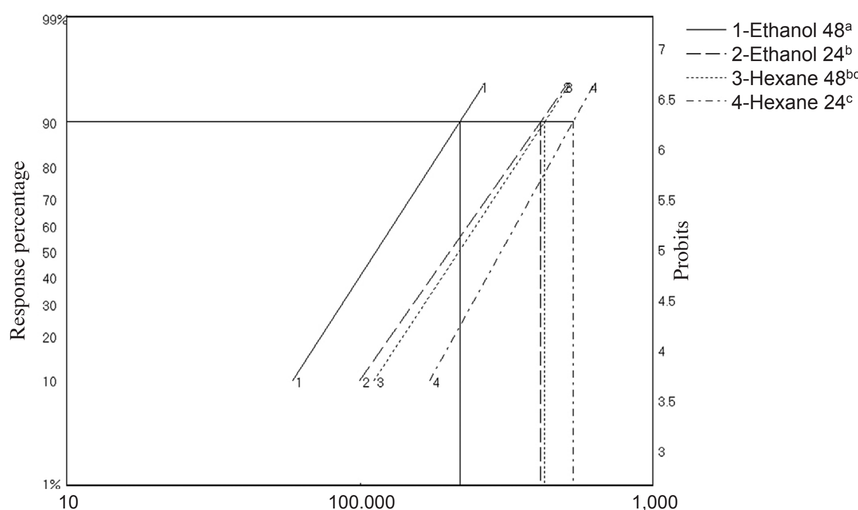

Fig 2–Graph showing LC_{90} values of ethanol and hexane crude extracts of *Steccherinum* sp mushroom against *Ae. aegypti* 3rd instar stage (n = 25) at 24- and 48-hour exposure. X-axis denotes extract concentration in ppm. Statistically significant differences are indicated by different letters (upper right).

val mortality after 24- and 48-hour exposure, respectively (Table 1). For 24-hour exposure, the highest activity was found from *Th. porentosus* (PHK27) extract with 70% mortality rate, following with *Steccherinum* (CKW03), *X. nigripes* (PW03) and GSW04 sample demonstrated 66%, 64% and 52% mortality, respectively, and after 48-hour exposure, larval mortality of 90%, 88%, 88%, and 70% mortality was obtained for *Steccherinum* sp (CKW03), *X. nigripes* (PW03), GSW04 and *Th. porentosus* (PHK27), respectively. Lower larvicidal activities (10% and 18% mortality for 24- and 48-hour exposure, respectively) were found with *Chlorophyllum* sp (NU01) and CKW05 samples, and the other 135 samples showed only 0 - 1% and 0 - 2% larval mortality after 24- and 48-hour exposure, respectively.

Based on the above data *Steccherinum* sp (CKW03) (10 g dried powder) was chosen for serial extraction with hexane, ethanol and water, producing a crude extract yield of 2.29, 8.58 and 18.59 g,

Table 1
Mushroom species and mortality rates of *Ae. aegypti* 3rd instar larvae (n=25) after exposure to 1% (w/v) mushroom aqueous extracts for 24 and 48 hours.

No.	Sample	Mushroom	Mortality (%) 24 h	Mortality (%) 48 h	No.	Sample	Mushroom	Mortality (%) 24 h	Mortality (%) 48 h
1	UMC 01	*Clitocybe* sp	0	0	42	UMC 42	*Earliella* sp	0	0
2	UMC 02	*Cantharellus cibarius*	0	0	43	UMC 43	*Stereum* sp	0	0
3	UMC 03	*Lentinus polychrous*	0	0	44	UMC 44	*Ganoderma applanatum*	0	0
4	UMC 04	*Abortiporus* sp	0	0	45	UMC 45	*Ganoderma lucidum*	0	0
5	UMC 05	*Schizophyllum commune*	0	0	46	UMC 46	*Earliella* sp	0	0
6	UMC 06	*Lentinus* sp	0	0	47	UMC 47	NI	0	0
7	UMC 07	*Lenzites vespacea*	0	0	48	UMC 48	*Ganoderma lucidum*	0	0
8	UMC 08	*Trametes hirsuta*	0	0	49	UMC 49	*Polyporus* sp	0	0
9	UMC 09	*Ganoderma* sp	0	0	50	UMC 50	*Scytinopogon angulisporus*	0	0
10	UMC 10	*Lentinus squarrosulus*	0	0	51	UMC 51	*Ganoderma lucidum*	0	0
11	UMC 11	*Macrocybe crassa*	0	0	52	UMC 52	*Podoscypha* sp	0	0
12	UMC 12	*Rigidoporus* sp	0	0	53	UMC 53	*Steccherinum* sp	0	0
13	UMC 13	*Bjerkandera* sp	0	0	54	UMC 54	*Fomes* sp	0	0
14	UMC 14	*Ganoderma* sp	0	0	55	UMC 55	*Ganoderma* sp	0	0
15	UMC 15	*Pycnoporus sanguineus*	0	0	56	UMC 56	*Ganoderma* sp	0	0
16	UMC 16	*Steccherinum* sp	0	0	57	UMC 57	*Fomes* sp	0	0
17	UMC 17	*Ganoderma lucidum*	1	1	58	UMC 58	*Ganoderma lucidum*	0	0
18	UMC 18	*Trametes* sp	0	0	59	UMC 59	*Ganoderma lucidum*	0	1
19	UMC 19	*Trametes* sp	0	0	60	UMC 60	*Lentinus polychrous*	0	0
20	UMC 20	*Microporus xanthopus*	0	0	61	UMC 61	*Lentinus polychrous*	0	0
21	UMC 21	*Podoscypha* sp	0	0	62	UMC 62	*Astraeus odoratus*	0	0
22	UMC 22	*Laccaria laccata*	0	0	63	UMC 63	*Lentinus squarrosulus*	0	0
23	UMC 23	*Macrocybe crassa*	0	0	64	UMC 64	*Amanita caesarea*	0	0
24	UMC 24	*Chlorophyllum molybdites*	0	0	65	UMC 65	*Russula rosacea*	0	0
25	UMC 25	*Trametes* sp	0	0	66	UMC 66	*Amanita princeps*	0	0
26	UMC 26	*Polyporus* sp	0	0	67	UMC 68	*Amanita princeps*	0	0
27	UMC 27	*Trametes* sp	0	0	68	UMC 69	*Auricularia auricular*	0	0
28	UMC 28	*Ganoderma lucidum*	0	0	69	UMC 70	*Lentinus squarrosulus*	0	0
29	UMC 29	*Meruliopsis* sp	0	0	70	UMC 71	*Ganoderma applanatum*	0	0
30	UMC 30	*Lenzites* sp	0	0	71	UMC 72	*Polyporus* sp	0	0
31	UMC 31	*Stereum ostrea*	0	0	72	UMC 73	*Ganoderma applanatum*	0	0
32	UMC 32	*Ganoderma* sp	0	0	73	UMC 74	*Pycnoporus sanguineus*	0	0
33	UMC 33	*Stereum* sp	0	0	74	UMC 75	*Ganoderma lucidum*	0	0
34	UMC 34	*Lenzites elegans*	0	0	75	UMC 76	*Polyporus* sp	0	2
35	UMC 35	*Ganoderma* sp	0	0	76	UMC 80	*Ganoderma* sp	0	0
36	UMC 36	*Ganoderma lucidum*	0	0	77	UMC 81	*Ganoderma* sp	0	0
37	UMC 37	*Trametes versicolor*	0	0	78	UMC 82	*Fomes* sp	0	0
38	UMC 38	*Macrocybe crassa*	0	0	79	UMC 83	*Ganoderma lucidum*	0	0
39	UMC 39	*Trametes* sp	0	0	80	UMC 84	*Ganoderma lucidum*	0	0
40	UMC 40	*Stereum* sp	0	0	81	UMC 85	*Microporus xanthopus*	0	0
41	UMC 41	*Trametes* sp	0	0	82	UMC 86	*Pycnoporus sanguineus*	0	0

Table 1 (Continued).

No.	Sample	Mushroom	Mortality (%)		No.	Sample	Mushroom	Mortality (%)	
			24 h	48 h				24 h	48 h
83	UMC 87	*Phaeolus* sp	0	2	114	AMC 13	*Geastrum saccatum*	0	0
84	UMC 88	*Microporus xanthopus*	0	0	115	AMC 15	*Dictyophora indusiata*	0	0
85	PHK 01	*Scleroderma polyrhizum*	0	0	116	AMC 16	*Tremella fuciformis*	0	0
86	PHK 02	*Boletus chromapes*	0	0	117	NU 01	*Chlorophyllum* sp	10	18
87	PHK 03	*Lactarius hatsudake*	0	0	118	NU 02	*Chlorophyllum* sp	0	0
88	PHK 04	*Boletellus emodensis*	0	0	119	NU 03	*Chlorophyllum molybdites*	1	1
89	PHK 05	*Fomitopsis pinicola*	0	0	120	NU 04	*Schizophyllum commune*	0	0
90	PHK 07	*Microporus* sp	0	0	121	CKW 01	*Stereum hirsutum*	0	0
91	PHK 08	*Amanita verna*	0	0	122	CKW 02	*Fomitopsis pinicola*	0	0
92	PHK 09	*Boletus* sp	0	0	123	CKW 03	*Steccherinum* sp	66	90
93	PHK 13	*Laccaria laccata*	0	0	124	CKW 04	*Boletus* sp	0	0
94	PHK 14	*Laccaria* sp	0	0	125	CKW 05	NI	10	18
95	PHK 17	*Termitomyces* sp	0	0	126	CKW 06	NI	0	0
96	PHK 21	*Hygrocybe* sp	0	0	127	STW 01	*Trametes* sp	0	0
97	PHK 22	*Scleroderma sinnamariense*	0	0	128	STW 02	*Fomitopsis* sp	0	0
98	PHK 23	*Armillaria* sp	0	0	129	STW 03	NI	0	0
99	PHK 24	*Polyporus* sp	0	0	130	MPW 01	*Cyathus striatus*	0	0
100	PHK 25	*Stereum ostrea*	0	0	131	MPW 02	*Mycena* sp	0	0
101	PHK 26	*Russula* sp	0	0	132	MPW 03	*Cyathus striatus*	0	1
102	PHK 27	*Thaeogyroporus porentosus*	70	70	133	MPW 04	*Thelephora penicillata*	1	2
103	AMC 01	*Russula rosacea*	1	1	134	PW 01	NI	0	0
104	AMC 02	*Termitomyces* sp	0	0	135	PW 02	NI	0	0
105	AMC 03	*Russula alboareolata*	0	0	136	PW 03	*Xylaria nigripes*	64	88
106	AMC 04	*Russula cyanoxantha*	0	0	137	GSW 01	*Scytinopogon angulisporus*	0	0
107	AMC 05	*Sentinus* sp	0	0	138	GSW 02	*Ganoderma* sp	0	0
108	AMC 06	*Amanita vaginata*	0	0	139	GSW 03	*Daedaleopsis confragosa*	0	0
109	AMC 07	*Russula densifolia*	1	1	140	GSW 04	NI	52	88
110	AMC 08	*Amanita princeps*	0	0	141	GSW 05	*Thelephora* sp	0	0
111	AMC 10	*Amanita hemibapha*	0	0	142	GSW 06	*Ramaria* sp	0	0
112	AMC 11	*Amanita princeps*	0	0	143	GSW 07	*Ganoderma* sp	0	0
113	AMC 12	*Russula* sp	0	0					

AMC and UMC samples were collected from Chiang Mai, Krabi, Lampang, Nakhon Ratchasima, Nakhon Sawan, Pathum Thani, Phichit, Phitsanulok, Phuket, Prachuap Khiri Khan, Sukhothai, Surat Thani and Tak Provinces, Thailand. CKW, GSW, MPW, NU, PHK, PW, and STW samples were collected only from Phitsanulok Province. NI, not identified.

respectively. Ethanol extract shows statistically lower LC_{50} values than hexane extract after 24-hour (203 ppm *vs* 304 ppm) (Fig 1) and 48-hour (114 *vs* 218 ppm) exposures (48-hour) (Fig 2 and Table 2). Similar phenomena were observed for LC_{90} values, which are statistically lower than LC_{50} values. However, the aqueous extract lacked larvicidal activity (up to 1,000 ppm) after 48-hour exposure.

Table 2
Larvicidal activities of hexane and ethanol *Steccherinum* sp extracts against *Ae. aegypti* 3rd instar larvae (n=25) after 24- and 48-hour exposure.

Steccherinum sp extract (ppm)	24-hour exposure					48-hour exposure				
	% mortality (mean±SE)	Larvicidal activity				% mortality (mean±SE)	Larvicidal activity			
		Lethal concentration with fiducial limits (ppm)					Lethal concentration with fiducial limits (ppm)			
		LC_{50}	LC_{90}	χ^2	Slope + SE		LC_{50}	LC_{90}	χ^2	Slope + SE
Hexane										
100	1 ± 1	304	533	11.56	4.8 ± 0.4	8 ± 2	218	426	0.14	4.4 ± 0.5
200	21 ± 3	(239 - 374)	(484 - 872)			42 ± 3	(201 - 239)	(366 - 533)		
300	41 ± 2					74 ± 4				
400	67 ± 2					100				
500	95 ± 2					100				
Control	0					1 ± 1				
Ethanol										
50	1 ± 1	203	413	10.49	4.2 ± 0.3	8 ± 2	114	220	7.1	4.5 ± 0.3
100	9 ± 2	(191 - 215)	(377 - 461)			37 ± 3	(106 - 122)	(201 - 245)		
150	25 ± 2					62 ± 5				
200	51 ± 4					91 ± 2				
250	72 ± 2					96 ± 2				
300	77 ± 2					100				
350	85 ± 2					100				
400	92 ± 2					100				
Control	0					0				

DISCUSSION

In this study we show, for the first time to the best of our knowledge, that Thai mushrooms in the genera *Thaeogyroporus* (*T. porentosus*, PHK27), *Chlorophyllum* sp (NU01), *Steccherinum* sp (CKW03) and *Xylaria* (*X. nigripes*, PW03) and 2 unidentified samples (CKW05 and GSW04) contain metabolites with *Ae. aegypti* mosquito larvicidal property. Using sequential extraction with hexane, ethanol and water, the ethanol extract showed superior larvicidal activity over that of hexane, and the aqueous extract lacked activity over the range of time and concentration tested.

Chelela *et al* (2014) reported that mushrooms in the genera *Amanita* (*A. phalloides*), *Boletus*, *Lactarius* (*L. densifolius*, *L. gymnocarpoides*) and *Russula* (*R. cellulata* and *R. kivuensis*) showed larvicidal activity. However, although we found these genera in our samples but they are of different species and showed little or no toxicity towards *Ae. aegypti* larvae. We did not find *Cyptotrama asprata* earlier reported by Njogu *et al* (2009).

Ethyl acetate extract of one species of the mushroom genus *Pycnoporus* (*P. sanguineus*) from Manaus, Brazil has a larvicidal activity against *Ae. aegypti* larvae with LC_{50} value of 156.8 ppm at 24-hour exposure (Bucker *et al*, 2013). However samples of this species from Phitsanulok, Chiang Mai and Krabi Provinces, Thailand showed no larvicidal activity. It is possible that different extraction techniques and/or different geographical locations and habitats might produce different bioactive components. In addition, whether the same morphologically identical mushroom species have the same genetic characteristics needs further investigation.

It should be noted that not all samples in the genera *Chlorophyllum* and *Steccherinum* showed larvicidal toxicity. We assume that different mushroom species could contain different bioactive substances. The species of our *Chlorophyllum* and *Steccherinum* samples must be investigated to support the assumption. In our study, limitations in identifying the species of *Steccherinum* samples was due to the limitation of obtaining the complete morphology as the specimens were soft and frail, and easily lost their natural form and characteristics during transportation. Alternative techniques, such as identification of molecular markers, should be used to identify mushroom species of interest. By using ribosomal DNA internal transcribed spacer sequences, identification of a number of mushroom species has been reported (Rajaratnam and Thiagarajan, 2012; Das *et al*, 2013; Olusegun, 2014).

In conclusion, the large mushroom diversity and presence of numerous species still to be identified provide a rich resource for discovery of natural larvicides as safer alternatives to synthetic chemical insecticides in current use to control mosquitoes.

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