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Research Article

Traditional Plant (Stemona collinsae, Curcuma longa and Bambusa multiplex) Use to Repel Blowfly Larvae in Fermented Fish (Pla-ra)

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Abstract

Background and Objective: Pla-ra or Pla-dek is a common local ingredient of Thai cuisine. During fermentation process, spoilage from bacteria or infestation with blowfly larvae can occur. Local herbs were studied for protection against attack by blowfly larvae in fermented fish. Three traditional Thai plant species *Stemona collinsae*, *Curcuma longa* and *Bambusa multiplex* were tested for blowfly repellence and larvicidal activity. **Materials and Methods:** After *Chrysomya megacephala*larvae exposed traditional Thai plant, results were recorded at 0, 1, 3, 6, 12 and 24 h after exposure and scanning electron microscopy for study the surface ultrastructure of the blowfly larvae post-treatment. **Results:** Highest repellent effect of *S. collinsae* placed on top of fermented fish was 74.44% within 1 h. This species was significantly more effective in a dose-dependent manner than other plant species and the control. *Bambusa multiplex* was more effective for larvicidal activity than the other plant species. Results showed that fermented fish added with *S. collinsae* had the highest potential repellent effect at r = 0.944, while *Bambusa multiplex* strongly inhibited larvicidal activity against blowfly larvae at r = 0.845. Surface tegumental microtopography by SEM showed wrinkled small vesicles with shrunken heads and end parts. **Conclusion:** This study demonstrated that plants are effective in repelling and killing blowfly larvae during the production process of fermented fish in factories and traditional fermentation in open jars. The result proved the hypothesis that plants traditionally used against blowfly larvae are effective in fermented fish production in the northeast of Thailand.

Key words: Blowfly, repellent, traditional plants, scanning electron microscope

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

Fish fermentation is widely used as a preservation process in Southeast Asia. In Northeastern Thailand, people catch fish for consumption that they preserve using salt. This is called Pla-ra or Pla-dek. Eating fermented fish is a possible risk factor of cholangiocarcinoma in Thailand¹. Pla-ra is a common local ingredient of Thai cuisine and well-liked by Isan people. During the fish catching season, people ferment residual fish by mixing with salt and rice under tropical temperatures for 1-2 years. However, after sealing the fermentation jar, spoilage from bacteria or infestation with blowfly larvae can occur. Non-biting flies in the family Calliphoridae comprise more than 1,000 species and some species in the genus *Chrysomya* are carriers of intestinal human diseases2. Blowflies or *Chrysomya megacephala* are important transmission vectors and can be found worldwide. In the fermented fish process that takes place in kitchens or factories, larvae and adults of blowfly species are commonly found and these pose a significant public health problem. Mechanical transmission of human intestinal parasites by non-biting files has not received adequate scientific attention^{3,4}.

Traditional knowledge concerning plants serves two main purposes as treatment for myiasis and repelling fly larvae from fermented fish. The most reported plant species used against fly larvae were cinnamon, lavender, bamboo, *Clausena anisata* and *Azadirachta indica*^{5,6} and Thai traditional plants used were bamboo, Norn-Tai-Yark and homegrown vegetables^{7,8}. Here, it was focused on three local plants as *Stemona collinsae*, *Curcuma longa* and *Bambusa multiplex*.

The root of *Stemona collinsae* (Stemonaceae) in Thai called Norn-Tai-Yark has long been used by people for killing parasitic worms. Phytochemical evaluation revealed that stemona alkaloids were the main effective ingredient comprising tuberostemospironine, stenine, stemoamide and stemoamine⁹. Traditional Chinese medicines have been used for their antitussive, anti-inflammatory and anti-parasitic properties against agricultural pests and insects^{10,11}.

Bamboo shoots are used as foodstuffs in traditional communities in Asian countries including Thailand¹². In traditional Northeastern Thailand cuisine, bamboo shoots are placed inside open fermenting fish jars. The cyanide content in sour bamboo shoots kills or repels fly larvae^{13,14}.

Curcuma longa or turmeric is a well-known traditional plant in Thailand. Turmeric rhizomes contain terpene class components such as α -turmerone and cineole. Therapeutic properties of turmeric include anti-inflammation and anti-microbial action. Turmeric oil acts as a repellent against mosquitos and cockroaches¹⁵.

Sealing the jars of fermenting fish is a challenge in rural areas and the main cause of spoilage is infestation with fly larvae. This study tested local traditional plant recipes for repelling and killing blowfly larvae (*Chrysomya megacephala*) during the fermenting fish process. Experiments were carried out using *S. collinsae*, *C. longa* and *B. multiplex* to test their effectiveness against blowfly larvae infestation by covering fermenting jars.

MATERIALS AND METHODS

Study area: The study was carried out at Department of Thai Traditional Medicine, Microbiology and Parasitology Lab from September, 2018-December, 2019.

Traditional plant preparation: Plant material of *Stemona collinsae*, *Curcuma longa* and *Bambusa multiplex* were obtained from Sakon Nakhon province in the Northeast of Thailand (Fig. 1). All plants were cleaned, peeled and cut into longitudinal slices of about 50-100 mm² similar to traditional use. After processing, the fresh plant materials were used immediately. Plants free from insects and lesions or other damage were selected and used following the method of De Boer *et al.* ⁶.

Blowfly egg cultivation: Morphological identification of adults of the blowfly species C. megacephala in the megacephala species group followed by previous study^{16,17}. Chrysomya megacephala were obtained from fresh markets in Phang Khon district, Sakon Nakhon (17°20'26"N 103°42′59″E), Thailand, using a catching net and then moved to a plastic cage and transported to the laboratory. Two hundred grams of fresh fish heads of Clarias batrachus catfish were placed on a tray and covered by a tent made of netting mesh near the bait tray. The trap was opened for 3 h, 8:00-11:00 am, as a suitable time for the female blowflies to locate the bait and lay eggs. After closing, blowfly adults and their eggs were identified by a parasitologist. Laboratory conditions for rearing were maintained at 24-28°C, 12:12 h (light:dark). The bait tray was moved to a sealed enclosure and left for 36 h for egg hatching and larval presentation¹⁷.

Experimental setup: The repellent efficacies of the three different plants were studied. The third-instar larvae grow to about 16 mm long and the posterior spiracles have 3 slits. The band of spinules on the last segment is incomplete dorsally 18. The anterior spiracle has 11-13 branches. About 10 third-instar larvae were randomly collected from the bait, transferred to containers and placed into 36 plastic cylindrical

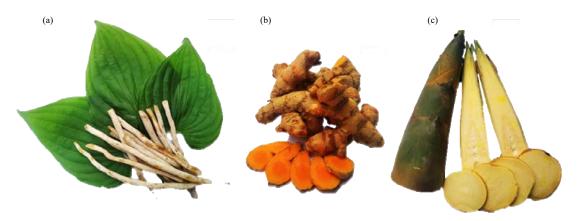


Fig. 1(a-c): Fresh plant (a) Stemona collinsae, (b) Curcuma longa and (c) Bambusa multiplex

sized 150×700 mm², 1 L jars containing 100 g of one-year-old fermented fish, then mixed with the three plant materials at 1:100 w/w and covered to test repellent activity. The control jars had no plant material added. The jars were placed in water plastic ware and observed every hour for 24 h. The numbers of escaped larvae were recorded. After 24 h, the total numbers of dead and surviving larvae were counted. Repellent activity of the plant materials was quantified as larvae leaving the fermented fish after adding plant materials and escaping from the jar by moving out from mesh and drowning in the water container. Numbers of larvae were measured each hour for up to 24 h. Larvicidal activity was defined as larvae dying inside the jar after placing the plant material, measured every hour for 24 h.

Scanning Electron Microscopy (SEM): To study the surface ultrastructure of the blowfly larvae post-treatment, 5 larvae from the highest effective treatment group were washed several times with 0.2 M cacodylate buffer (pH 7.2) and fixed with Karnovsky's solution at 4°C overnight. After washing for 10 min, 3 times with the phosphate buffer, samples were post-fixed with 1% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer (pH 7.2) for 1 h, followed by washing again 3 times for 10 min in phosphate buffer. Samples were dehydrated through a graded ethanol series (30, 50, 70, 90, 95 and 100% alcohol, 2 times), dried with a critical point dryer, coated with gold using a JFC-1100E ion sputtering device (JEOL, Tokyo, Japan) and observed with a JEOL JSM-7800F Scanning Electron Microscope (SEM) at 1.0 kV accelerating voltage.

Statistical analysis: Data for mortality levels and repellency for larvae in each period were submitted for variance analysis (ANOVA) followed by mean comparisons using Tukey's test at

5% significance level and Spearman's correlation test. All statistical analyses were performed with SPSS (version16.0).

RESULTS

Repellent and larvicidal activities: For repellent assay, the number of escaped larvae from each fermented fish jar was recorded every hour for up to 24 h. Percentages of escaped blowfly larvae for each treatment depended on the repellent effect of the plant species and concentration. Results in Fig. 2a show a strong correlation for the repellent effect from *S. collinsae* (100% escaped at 24 h) followed by *C. longa* (Fig. 2b) and *B. multiplex* (Fig. 2c) at 83-86 and 55-64%, respectively. For plant material added in fermented fish jars, the highest number of dead blowfly larvae was shown by *B. multiplex* at 47.4% with significance of Spearman's rank correlation followed by *C. longa* and *S. collinsae* at 5.6±2.4 and 0.0±0.0%, respectively as shown in Table 1.

Total repellence and larvicidal activities are shown in Table 1, with highest impact of *S. collinsae* (100 ± 0) for *S. collinsae* or Norn-Tai-Yark, the larvicidal activity no appears but the repellence higher. The *B. multiplex* was more effective for larvicidal activity than the other 2 plant species. Total effects of all plant species did not greatly differ. Overall results of Spearman's rank correlation and efficacy of the plant materials for repellence and larvicidal activities of Norn-Tai-Yark, *B. multiplex* and *C. longa* were 0.944, 0.754, 0.774 and 0.789, 0.845, 0.826, respectively as shown in Table 2.

Surface changes by Scanning Electron Microscopy (SEM):

The larva body of *Chrysomya megacephala* after incubation in the fermented fish jars as shown in Fig. 3a-e. In the control

Table 1: Repellence, larvicidal activity and total effect of each treatment added at 24 h on Chrysomya megacephala larvae

Species	No.	Escaped	Dead	Total
Control	9	5.9±2.8	00.0±0.0	5.9±2.8
S. collinsae	9	10.0 ± 0.0	00.0 ± 0.0	10.0±0.0**
C. longa	9	78.1±7.1	5.6±2.4	83.7±6.5**
B. multiplex	9	39.7±9.3	47.4±5.2	87.1±9.8**

Significant values **p<0.001 for total effect between control and each treatment

Table 2: Correlation of traditional plants added at 10, 20 and 30 g and total number of escaped and dead Chrysomya megacephala larvae

Species	No.	Escaped		Dead	
		Spearman's Rho	p-value	 Spearman's Rho	p-value
S. collinsae	9	0.944	0.09	0.789	0.012
C. longa	9	0.774	0.014	0.826	0.045
C. longa B. multiplex	9	0.754	0.019	0.845	0.040

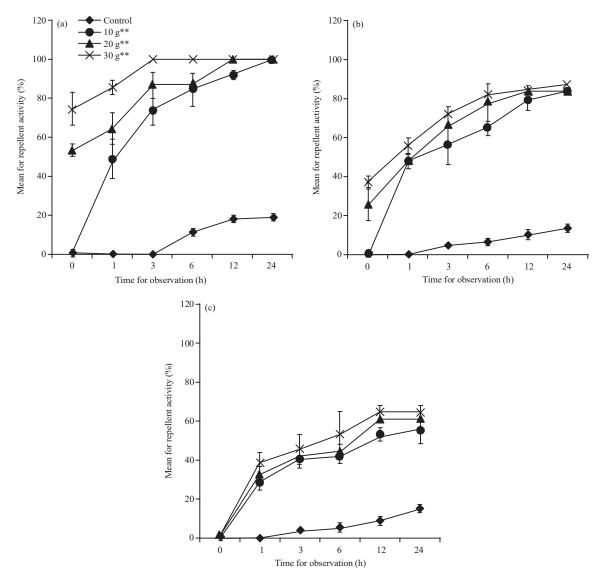


Fig. 2(a-c): Repellence of blowfly larvae in fermented fish over a 24 h period (average percentage of 3 replicates), **p<0.001 indicates statistical difference between the control and treatment groups (a) *Stemona collinsae*, (b) *Curcuma longa* and (c) *Bambusa multiplex* at all observation times

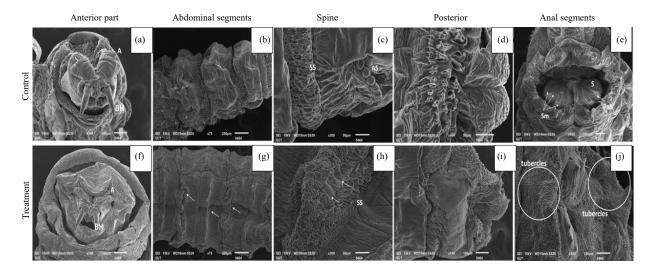


Fig. 3(a-j): Scanning electron micrographs of blowfly larvae, (a-e) Control group, (b-c) Normal appearance with regular spines (b and c) in segments, (f-j) For treatment groups, (f,g) Head and abdominal segments are shrunk and (i and j) Wrinkled (arrow) and posterior body, shows bleb tubercles formation

A: Antenna, As: Anterior spiracle, SS: Segmental spine, Sm: Spiracular muscles, S: Slit

group, larvae appear normal with the cephalic region composed of a pair of antennae and the maxillary palps and mouthparts composed of buccal hooks (Fig. 3a). Abdominal segments are flattened with sharp intersegmental spines (Fig. 3b), a clear anterior spiracle is located on the first thoracic segment (Fig. 3c) and posterior spiracular discs are stationed in a shoal cavity (Fig. 3d). Three spiracular slits are located in each of the posterior spiracular discs (Fig. 3e). Surface of the larvae changed after treatment and exhibited an abnormal appearance. The head section shrunk backward into the body (Fig. 3f) dilatation in abdominal segments (Fig. 3g) and spine shortening(Fig. 3h). Most tubercles appeared in the posterior section as shown in Fig. 3i and j.

DISCUSSION

Three Thai traditional plant species as *S. collinsae*, *C. longa* and *B. multiplex* displayed significant repellent activity against blowfly larvae. For this study, results supported local informants who reported the traditional use of Norn-Tai-Yark to inhibit blowfly larvae in fermenting fish jars. Norn-Tai-Yark showed more repellence than the other two species. By contrast, *B. multiplex* strongly impacted larvicidal activity. Total repellence and larvicidal activity for all plant species were similar. Results showed that all traditional plant species had indifferent effects as fermented fish jar additives. However, *B. multiplex* recorded more than 50% of dead fly

larvae in fermented fish jars. Both dead and living blowfly larvae are removed before consumption or sale in the market. By contrast, the use of *S. collinsae* on the top or inside fermented jars at maximum concentration can repel 100% of blowfly larvae within 3 h and is suitable for use in the fermentation process.

In Norn-Tai-Yark, the main active compounds are alkaloid insecticides as stemofoline¹⁹, tuberostemoline, 1,9-epoxy-9ahydroxystenine and protostemonine^{20,21}. Thai traditional knowledge has historically been used for pesticide control. A previous study by Brem et al. 22 found that S. collinsae root displayed very high insect toxicity^{7,23}. Repellence mechanisms for *S. tuberosa* alkaloids are similar to nicotine (alkaloid group) activity by activating acetylcholinesterase inhibitory activity that impacts muscle coordination and affects the nervous system inducing neurotoxic symptoms ^{24,25}. The present study, findings in ultrastructure indicated that *S. tuberosa* showed contraction of the larval body segments²⁶. This study represented the first report concerning repellence of blowfly larvae in fermented fish jars using *S. collinsae*. Larvae showed shrunken cephalic parts and dilated body segments with tubercles in the posterior body as observed in the SEM results (Fig. 3f-j).

Bamboo species are a novel source of nutrition and medicine. They are used in Laos in the fermented fish process, but not in other Asian countries⁶ and bamboo shoots are cooked for consumption²⁷. Raw bamboo shoots contained

taxiphyllin, a cyanogenic glycoside that has side effects on human health²⁸. When cyanogenetic glycosides are hydrolyzed by body processes, they emit hydrogen cyanide. This is extremely toxic and can inhibit the enzyme cytochrome oxidase, resulting in cellular disruption that may be fatal. Cyanoglycosides can interfere with larvae contraction of the irritated segment leading to mortality. Our results showed more than 50% of larvicidal activity by fresh *B. multiplex* added to fermented fish jars^{29,30}. However, bamboo shoots are suitable for human consumption after boiling when removed from the fermented fish.

Turmeric or *Curcuma longa* is widely distributed in the tropical regions of Southern Asia including Thailand. Turmeric rhizomes are a rich source of curcumin, cineole and camphor. Turmeric has long been used as a food ingredient and also for treatment of gastrointestinal disorders³¹, inflammatory bowel disease³² and antiatherosclerotic effects³³. In this study, fresh turmeric showed $83.7\pm6.5\%$ total repellence and larvicidal activities. Our results were supported by Liu *et al.*³⁴. Camphor is an essential oil in turmeric rhizomes that exhibits insecticidal activity³⁴. The mechanism of volatile insecticides is presently unknown. However, possible modes of action include neurological activity and respiratory inhibition via mitochondrial impacts that induce paralysis-like symptoms and immobility^{35,36}.

This research showed that Norn-Tai-Yark root was the most effective for repelling blowfly larvae followed by turmeric and bamboo. Fresh bamboo shoots (*B. multiplex*) gave high correlation for killing blowfly larvae followed by turmeric (*C. longa*).

CONCLUSION

Our results proved the hypothesis that plants traditionally used against blowfly larvae are effective in fermented fish production in the Northeast of Thailand. This is the first report that examines the surface change in larvae body in fermentation fish jars after treatment.

SIGNIFICANCE STATEMENT

This study evaluated traditional knowledge on plant use to repel blowfly larvae and investigate larvae body change by SEM method after added *Stemona collinsae*, *Curcuma longa* and *Bambusa multiplex* in fermenting fish jars and support Thai local informants who reported the traditional use of plant to inhibit blowfly larvae.

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