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Research article

Potential of Tobacco Extracts in Controlling Stable Flies (*Stomoxys calcitrans* L.) in Beef Cattle

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Abstract: This research aimed to evaluate the potential of tobacco extract for controlling stable flies (*Stomoxys calcitrans* L.) in beef cattle. The study was divided into three experiments: 1) determination of nicotine concentration in tobacco extract using different fermentation times, 2) laboratory assessment of tobacco extract efficacy against stable flies, and 3) field evaluation of tobacco extract efficacy against stable flies. Results showed that fermenting tobacco with 95% ethanol for 48 hours yielded the highest nicotine content at 46.71 μg/mg, which was significantly different ($P<0.001$) compared to other fermentation periods. Laboratory studies revealed that the efficacy of tobacco extract in eliminating stable flies increased with concentration and exposure time. The 20% tobacco extract showed maximum efficacy with mortality rates of 98.33% at 24 hours and 100% at 48 hours, while 3% tobacco extract achieved 95% mortality of stable flies at 72 hours. In field trials, 3% tobacco extract demonstrated significantly better control of stable flies compared to the control treatment (distilled water) ($P<0.001$), but was less effective than 35% cypermethrin. The efficacy of tobacco extract decreased after 96 hours post-application, corresponding with increased fly-repelling behaviors in cattle, including tail flicking, skin twitching, foot stamping, and head swinging. Results from this study indicate that tobacco extract has potential as an alternative to synthetic chemicals for controlling stable flies in beef cattle, which helps reduce chemical use and is environmentally friendly.

Keywords: Tobacco extract; Nicotine; Stable fly; Beef cattle; Biological insecticide

1. Introduction

Stable flies (*Stomoxys calcitrans* (L.) [Diptera: Muscidae]) cause significant economic damage to the livestock industry by irritating and inflicting painful bites on animals as they feed on their blood [1]. Several species of stable flies exist, but *S. calcitrans* L. is the most prevalent in Thailand [2]. According to Arjkumpa et al. [3], blood-feeding by stable flies can reduce cattle weight by 0.22 kg per day and decrease milk production by 30-40 percent. Additionally, stable flies serve as vectors for lumpy skin disease, an emerging disease first detected in Thailand in 2021. This severe disease results in morbidity rates of 5-45% in infected animals, with mortality rates being particularly high in regions where the disease had not previously occurred [4].

Farmers typically control stable flies using synthetic chemicals, which may adversely affect the health of both animals and users [5]. Therefore, natural

insecticidal extracts, such as tobacco extract, represent an enjoyable alternative to chemical pesticides [6]. Natural extracts offer several advantages, including specificity in targeting insects [7], low toxicity, rapid degradation, and minimal environmental impact [8]. Tobacco extract contains nicotine as its primary active compound, which is a natural alkaloid that acts on insect nervous systems through both contact and ingestion, demonstrating high efficacy against insects. Currently, these compounds are effectively used to eliminate household pests like ants, termites, and cockroaches [9].

In 2022, the Sa Kaeo Area Excise Office seized a large quantity of illegal tobacco products, including 165,959 packs of smuggled or counterfeit cigarettes. On May 10, 2023, 57,743 packs from finalized legal cases were officially transferred to Valaya Alongkorn Rajabhat University under the Royal Patronage, Sa Kaeo, for use in agricultural research. These seized cigarettes, previously designated for destruction, were repurposed to study their potential as botanical insecticides in line with the Zero Waste concept and efforts to reduce chemical pesticide use, minimize production costs, and avoid harm to humans, animals, and the environment. Therefore, this study aimed to (1) determine the nicotine concentration in tobacco extracts after different fermentation durations, (2) evaluate their insecticidal efficacy against stable flies under laboratory conditions, and (3) assess their effectiveness in controlling stable flies in beef cattle under field conditions in Khok Sung District, Sa Kaeo Province.

2. Materials and Methods

In this experiment, tobacco was provided by the Sa Kaeo Area Excise Office. This tobacco came from illegal contraband that had completed legal proceedings and was authorized for destruction by the Sa Kaeo Area Excise Office, according to official memorandum no. 0605/1162 dated April 24, 2023, regarding auction authorization and minimum pricing determination for confiscated items under the authority of the Excise Department of Thailand. The study on the efficacy of tobacco extract for controlling stable flies in beef cattle was divided into three experiments: 1) determination of nicotine concentration in tobacco extract using different fermentation times, 2) laboratory assessment of tobacco extract efficacy against stable flies in beef cattle, and 3) field evaluation of tobacco extract efficacy against stable flies in beef cattle (Animal use license number U1-03142-2559). The details are as follows:

2.1 Study of nicotine concentration in tobacco extracts using different fermentation times

The tobacco used in this study was obtained from confiscated cigarettes provided by the Sa Kaeo Area Excise Office. Although the exact plant parts used in the cigarettes were not specified, commercial cigarettes mainly contain tobacco leaves. Tobacco was unwrapped from its paper covering, weighed to 500 grams, and fermented with 500 milliliters of 95% ethanol [10] for periods of 48, 72, 96, and 120 hours at 27±1°C and 60-70% relative humidity under a 16:8 h light: dark cycle. The experiment followed a completely randomized design (CRD) with three replications. After completing the fermentation period, the mixture was filtered through a 10-micron filter cloth. The filtered solution was then evaporated to separate the solvent using a rotary evaporator (BUCHI R-114, BUCHI Corp., USA) at 65°C. The tobacco extract obtained was weighed to determine the yield in comparison to the initial tobacco weight. The prepared tobacco extracts were stored in amber glass bottles at 4°C in darkness to maintain stability and prevent degradation. Storage stability testing was conducted by monitoring the physical characteristics (color, pH, viscosity) and nicotine content of tobacco extracts at various concentrations stored under these conditions for up to 240 days, with analysis performed at intervals of 0, 15, 30, 60, 120, and 240 days. Results demonstrated good shelf-life stability with minimal degradation when stored under proper conditions.

Tobacco extract samples from fermentation periods of 48, 72, 96, and 120 hours were analyzed for nicotine content using High Performance Liquid Chromatography (HPLC) (Waters, Co., Ltd., USA). Nicotine analysis followed a method modified from Mihranyan [11], using a Symmetry C-18 column, 150 mm×3.90 mm, 5 μm particle size (Waters, Co, Ltd.). The mobile phase consisted of 0.1% (v/v) triethylamine in water: acetonitrile (70:30 v/v) with a flow rate of 1.0 milliliter per minute, detected by a UV Detector (Waters 486 Tunable Absorbance Detector) at a wavelength of 260 nanometers. This experiment used (-)-Nicotine hemisulfate salt ≥95% (TLC) 40% (w/v) as a standard, preparing standard solutions in methanol at concentrations of 10, 20, 40, 60, 90, and 100 μg/mL. HPLC analyzed the standard solutions to create a standard

curve between concentration and peak area. Nicotine content in each treatment's tobacco extract was calculated using the standard curve. Data from each treatment were analyzed according to the experimental design, and means were compared using Duncan's New Multiple Range Test at a 95% confidence level ($P<0.05$).

2.2 Efficacy study of tobacco extracts in controlling stable flies in beef cattle in laboratory conditions

Stable flies *(Stomoxys calcitrans* L.) were collected using sweep nets from a beef cattle farm (GPS coordinates: 13°46'20.2"N 102°10'59.6" E) at Valaya Alongkorn Rajabhat University under the Royal Patronage, Sa Kaeo Province. The stable fly was identified based on morphological characteristics under a high-magnification stereomicroscope. Diagnostic features included the presence of a forward-projecting proboscis with piercing-sucking mouthparts, clear wings with distinct venation, and a checkered black pattern on the abdomen. The body length was approximately 5-7 mm. The flies were raised in a laboratory in cages measuring 20×30×20 centimeters, made from wire frames covered with white nylon fabric with a 30-centimeter flap serving as an entrance-exit. The environment inside the breeding cage was arranged to simulate natural conditions by placing grass in plastic bags with water in plastic containers. Fresh cattle blood on cotton was provided as food daily. Fermented grass that had undergone a 7-day fermentation process was placed in a circular plastic container (5.5×1 centimeters in diameter) to serve as an egg-laying site for stable flies.

When the eggs hatched into larvae, they were raised in plastic containers. The larval diet, modified from Bailey et al. [12], consisted of wheat bran, bagasse, and water in a ratio of 3:1:5, placed in circular plastic containers (3.1×1.9 centimeters in diameter). Cotton circles with a diameter of 3 centimeters were moistened and placed on the food surface to prevent drying. The larval rearing containers were kept in a temperature-controlled cabinet at 25 ± 1°C with a relative humidity of 76-84%. Upon pupation, the pupae were transferred to circular plastic containers of the same size as the larval containers, covered with nylon fabric. When the pupae developed into adults, they were used in experiments.

Laboratory bioassays were conducted using 3-5 day old adult stable flies after emergence from pupae. The sex ratio of flies used in testing was not predetermined, maintaining natural proportions from the colony. No starvation period was applied before bioassay testing. During the observation period, treated flies were maintained in cages measuring 20×30×20 centimeters under controlled environmental conditions. Fresh cattle blood soaked in cotton and water were provided as food sources in circular plastic containers (4 cm diameter × 3 cm height) placed inside each cage to ensure fly survival during the observation period. All bioassay experiments were conducted under controlled laboratory conditions at 27±1°C and 60-70% relative humidity under a 16:8 h light:dark cycle.

The experiments involved spraying tobacco extract from the previous experiment that yielded the highest nicotine content. The extract was adjusted to concentrations of 1%, 3%, 5%, 10%, and 20% (w/v). Emulsifiers, including 10 grams of Tween 80 followed by 10 grams of Span 80, were added to increase product stability, and the mixture was homogenized. Then, 100 grams of water was added to the mixture and homogenized with a homogenizer [13]. The treatments were compared with distilled water (control) and a commercial stable fly insecticide (35% w/v cypermethrin, used at a rate of 10 milliliters per 20 liters of water).

The direct spray method was used with a high-pressure water sprayer equipped with a hollow cone nozzle (Disc and Core). The spray nozzle was positioned approximately 15 centimeters from the insects, and 0.5 milliliters of extract solution was evenly distributed. The experiment followed a completely randomized design (CRD) with three replications, using 20 stable flies per concentration. The experimental treatments were as follows:

Treatment 1: Distilled water (control)
Treatment 2: 35% cypermethrin (w/v) (used at a rate of 10 milliliters per 20 liters of water)
Treatment 3: 1% tobacco extract (w/v)
Treatment 4: 3% tobacco extract (w/v)
Treatment 5: 5% tobacco extract (w/v)
Treatment 6: 10% tobacco extract (w/v)
Treatment 7: 20% tobacco extract (w/v)

Data on stable fly mortality was recorded at specific time intervals of 1, 3, 6, 12, 24, 48, and 72 hours post-treatment. Flies were considered dead when they showed no movement after gentle prodding with a fine brush. Mortality rates were calculated and the mortality rate data was statistically analyzed at a 95% confidence level, with differences compared using Duncan's New Multiple Range Test (DMRT), and LC_{50} values determined by Probit analysis.

2.3 Efficacy Study of Tobacco Extracts in Controlling Stable Flies in Beef Cattle in Field Conditions

The study was conducted at a small-scale farm located in Moo 2, Non Mak Mun Subdistrict, Khok Sung District, Sa Kaeo Province. Field trials were conducted under average meteorological conditions of 26°C temperature, 60-65% relative humidity, no rainfall, with southeast winds at 10-30 km/h. Twelve native-Brahman crossbred beef cattle were used, divided into three groups of four animals each. Cattle were randomly allocated to treatments using a random number table, with each animal assigned a number from 1 to 12 and randomly distributed to ensure equal representation across treatments. The animals were kept in individual pens. The experiment followed a completely randomized design (CRD) with three treatments as follows:

Treatment 1: Distilled water (control)

Treatment 2: 35% cypermethrin (w/v) (used at a rate of 10 milliliters per 20 liters of water)

Treatment 3: Tobacco extract that demonstrated the best mortality effect on stable flies in laboratory tests

All three treatments were applied by direct spraying on the pen floor and the cattle using a high-pressure water sprayer equipped with a hollow cone nozzle (Disc and Core).

The stable fly population on the front legs of the cattle was counted after spraying all three treatments at 24, 48, 72, 96, and 120 hours, following the method of Mullens & Peterson [14]. An expert counted the flies visually on the front legs from below the upper leg joint downward, with a survey time of 3 minutes per animal. Data on the stable fly population per individual cow were recorded.

Additionally, fly-repelling behaviors of the cattle were studied after spraying all three treatments at 24, 48, 72, 96, and 120 hours. The frequency of four repelling behaviors was recorded: Tail flicking, skin twitching, Foot stamping, and head swinging, for 2 minutes per animal, following the method of Mullens et al. [15]. The frequency of all four repelling behaviors was recorded.

The collected data were statistically analyzed using analysis of variance in a completely randomized experimental design, and the differences between means were analyzed using Duncan's New Multiple Range Test at a 95% confidence level ($P<0.05$).

3. Results and Discussion

3.1 Determination of nicotine concentration in tobacco extract using different fermentation times

The HPLC method for nicotine quantification was validated with appropriate analytical performance. The calibration curve showed strong linearity ($R^2 = 0.9991$) across the concentration range of 77.2-619.6 μg/ml. Method precision was assessed through replicate analyses, demonstrating excellent repeatability with relative standard deviation (RSD) values ranging from 0.21% to 0.63% (average 0.44%) for all fermentation treatments, which is well within acceptable limits (<2.0%). The extraction yield of tobacco extract varied significantly with fermentation time. The highest extraction yield was obtained after 48 hours of fermentation (12.4 ± 0.3%), followed by 72 hours (11.8±0.2%). More extended fermentation periods showed decreased yields, with 96 hours yielding 10.2 ± 0.4% and 120 hours yielding 9.6±0.3% (Table 1). The study found that the fermentation time significantly affected the nicotine content in tobacco extracts ($P<0.001$). Fermentation for 48 hours yielded the highest nicotine content at 46.71 ± 0.22 μg/mg, which was significantly different compared to other fermentation periods. This was followed by fermentation for 72 hours, which produced a nicotine content of 42.76 ± 0.09 μg/mg. More extended fermentation periods resulted in significantly decreased nicotine content, with fermentation for 96 and 120 hours yielding only 25.07 ± 0.16 and 21.44 ± 0.10 μg/mg, respectively (Table 1).

These results demonstrate that the nicotine content in tobacco leaf solution tends to decrease as fermentation time increases, especially after 72 hours, when the nicotine content decreases significantly. The decrease in both extraction yield and nicotine content with extended fermentation time may be attributed to

the degradation of nicotine and other extractable compounds during prolonged exposure to ethanol and potential oxidation processes. Li et al. [16] studied tobacco fermentation at 60, 90, 120, 150, and 180 hours using 20 cigarettes with 100 ml of hexane solution. They found that the highest nicotine extract content was 115 µg/mL at 60 hours, with a decreasing trend to 106.55 µg/mL at 180 hours. Similarly, Tassew & Chandravanshi [17] found a nicotine content of 4.2% when extracting tobacco leaves fermented with acid-base for 30 hours. Furthermore, fermentation of tobacco with methanol and ethanol yielded 17.91-19.55% content at fermentation periods of 48-60 hours [18]. Therefore, the 48-hour fermentation period was considered appropriate for further insect testing.

Table 1. Extraction yield and nicotine content from fermentation at 48, 72, 96, and 120 hours.

Fermentation time (hours)	Extraction yield (%)	Nicotine content in tobacco solution (µg/mg)
48	12.4 ± 0.3^{a}	46.71 ± 0.22^{a}
72	11.8 ± 0.2^{b}	42.76 ± 0.09^{b}
96	10.2 ± 0.4^{c}	25.07 ± 0.16^{c}
120	9.6 ± 0.3^{d}	21.44 ± 0.10^{d}
P-value	<0.001	<0.001

[a-d] Means in the same column with different superscripts are significantly different (P<0.001).

3.2 Efficacy study of tobacco extracts in controlling stable flies in beef cattle in laboratory conditions

The study of the efficacy of various concentrations of tobacco extracts in controlling stable flies in beef cattle, when tested in laboratory conditions, showed that the mortality rates of stable flies differed with high statistical significance (P<0.001) across all testing intervals. Treatment 2 (35% cypermethrin), a synthetic chemical, provided the highest fly control efficacy with a mortality rate of 53.33% at 1 hour, increasing to 100% after 24 hours. For tobacco extracts, the efficacy in eliminating flies increased with concentration and exposure time. The 20% tobacco extract (Treatment 7) provided the highest efficacy among natural extracts, with a mortality rate of 41.66% at 1 hour, increasing to 98.33% at 24 hours, and 100% at 48 hours. This was followed by 10% tobacco extract (Treatment 6) and 5% tobacco extract (Treatment 5), both achieving 100% mortality at 72 hours. Meanwhile, 3% tobacco extract (Treatment 4) showed 95% mortality at 72 hours, and 1% tobacco extract (Treatment 3) demonstrated the lowest efficacy with only 70% mortality even after 72 hours. Treatment 1 (water), which served as the control, showed very low mortality throughout the experiment, with only 6.67% mortality at 72 hours, which was significantly different from all other treatments (Table 2).

Generally, the amount of nicotine extracted from tobacco leaves ranges between 2%-6% [19], which aligns with Tayoub et al. [20], who measured nicotine content in the dry weight of five different tobacco leaves using HPLC and LS-MS techniques, yielding approximately 3.3% to 6.7%. Djapic et al. [21] extracted nicotine from two types of tobacco leaves using the SC-CO_2 extraction technique, with nicotine extraction ratios to dry weight of raw materials equaling 2.33% and 2.99%, respectively.

From the study of stable fly mortality rates, the results demonstrated that tobacco extracts at concentrations of 5-20% have potential for controlling stable flies in beef cattle, especially at 20% concentration, which provided efficacy comparable to the synthetic chemical cypermethrin 35% as contact time increased. Progressive increases in mortality rates over time were observed across all tobacco extract treatments, as demonstrated in Table 2, where mortality percentages consistently increased from 1-hour to 72-hour exposure periods. For example, a nicotine concentration of 25.2 mg/L showed LC_{50} mortality of stable flies at 24 hours after testing, with the mortality rate increasing after just 1 hour. Additionally, the results indicated that nicotine efficacy improves with increased quantity and contact time. Therefore, insect mortality rates are influenced by both concentration and time, consistent with the toxicity of essential oils extracted from various plants such as tea tree oil (*Melaleuca alternifolia*), catnip (*Nepeta cataria*), and Indian borage (*Plectranthus amboinicus*). When tested through contact and vapor methods against stable flies at a quantity of 20 milligrams, these essential oils achieved 100% mortality within 19 minutes [22-24]. This aligns with Kanmani et al. [25], where nicotine extract was the main active ingredient in eliminating adult *Sitophilus oryzae*. After 24 hours of spraying, 100% mortality of adults was observed with an extract concentration of 5.00 mg/L. Nicotine extract showed the highest percentage of adult mortality at the lowest concentration, with LD_{50} and LD_{90} values of

1.62 and 2.85 mg/L, respectively. This trend remained consistent after 48 and 72 hours of exposure. It has been reported that increased active ingredients can kill more insects at higher concentrations and longer exposure times.

According to reports on the active ingredients of tobacco leaves against pest insects, nicotine is one of the alkaloids extracted from plants that acts rapidly on the nervous system to eliminate insects [26], causing severe tremors, seizures, followed by paralysis. Additionally, nicotine inhibits the functioning of the nervous system, causing an imbalance in neural function. Stimulation of the nervous system is one of the most important mechanisms of traditional insecticides such as organophosphates and carbamates [27].

This research also noted that besides the action of nicotine in eliminating stable flies, factors affecting nicotine extract include the method used to apply substances to contact the insects, which affects the amount of insecticide received, causing differences in efficacy. Insect mortality rates depend on whether they receive a single dose or continuous exposure over a period. Additionally, the distribution and particle size of substances are important factors affecting the amount absorbed by insects and the permeation rate of pesticides into the insects. Moreover, the efficacy of extracts varies according to the sex of the flies. Langai, Muthomi & Mbega [28] reported that male houseflies (*Musca domestica*) and green bottle flies (*Chrysomya megacephala*) are more susceptible to eucalyptol than females because they are typically smaller. However, our study did not evaluate the influence of sex on insecticide susceptibility.

Table 2. Efficacy of Tobacco Extracts in Controlling Stable Flies in Beef Cattle in Laboratory Conditions.

Treatment	Mortality Rate (%)						
	1 hour	**3 hours**	**6 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
Treatment 1	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.67^{a}	3.33^{a}	6.67^{a}
Treatment 2	53.33^{f}	70.00^{e}	90.00^{f}	95.0^{e}	100.00^{f}	100.00^{e}	100.00^{c}
Treatment 3	11.67^{b}	21.67^{b}	31.67^{b}	41.67^{b}	50.00^{b}	58.33^{b}	70.00^{b}
Treatment 4	16.67^{bc}	31.67^{b}	43.33^{c}	56.67^{c}	71.67^{c}	81.67^{c}	95.00^{c}
Treatment 5	25.00^{cd}	43.33^{c}	60.00^{d}	71.67^{d}	81.67^{d}	91.67^{d}	100.00^{c}
Treatment 6	1.67^{d}	55.00^{d}	66.67^{d}	78.33^{d}	90.00^{de}	95.00^{de}	100.00^{c}
Treatment 7	41.66^{e}	66.67^{e}	78.33^{e}	90.00^{e}	98.33^{ef}	100.00^{e}	100.00^{c}
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a-f}$ Means in the same column with different superscripts are statistically significantly different.
Treatment 1: Distilled water (Control), Treatment 2: 35% Cypermethrin, Treatment 3: Tobacco extract 1% concentration, Treatment 4: Tobacco extract 3% concentration, Treatment 5: Tobacco extract 5% concentration, Treatment 6: Tobacco extract 10% concentration, Treatment 7: Tobacco extract 20% concentration

Furthermore, the LC_{50} of tobacco extract that can cause 50% mortality of stable flies within 12 hours is a concentration of 3% or higher. Treatment 4 using 3% extract concentration showed a mortality rate of 56.67%, exceeding the specified 50% threshold. LC_{50} and LC_{90} values decreased significantly with increasing exposure time, from 40.56% (95% CI: 21.51-76.49%) at 1 hour to 0.40% (95% CI: 0.04-4.26%) at 72 hours. Similarly, LC_{90} values showed a dramatic reduction from 1850.27% (95% CI: 362.27-9450.02%) at 1 hour to 1.69% (95% CI: 0.46-6.16%) at 72 hours. The R^2 values ranged from 0.8166 to 0.9862, indicating strong dose-response relationships across all time intervals. At 12 hours, the LC_{50} was 1.73% (95% CI: 1.15-2.60%) and the LC_{90} was 23.17% (95% CI: 13.89-38.66%), confirming that the 3% concentration selected for field studies provides adequate control efficacy (Table 3). Although higher concentrations demonstrated better efficacy in eliminating stable flies, they may result in higher production costs or increased environmental risks. Therefore, the research team selected the 3% extract concentration (Treatment 4), which can control stable flies according to the specified criteria, for further study of nicotine efficacy in controlling stable flies in field conditions.

Table 3. Lethal Concentration (LC_{50} and LC_{90}) of Tobacco Extract (Treatment 4: 3% concentration) Against Stable Flies with 95% Confidence Intervals.

Time (hours)	LC_{50} (%)	LC_{50} 95% CI	LC_{90} (%)	LC_{90} 95% CI	R^2
1	40.56	21.51 - 76.49	1850.27	362.27 - 9450.02	0.9787
3	7.55	5.93 - 9.60	164.56	74.39 - 364.03	0.9862
6	3.37	2.45 - 4.66	67.48	29.56 - 154.02	0.9750
12	1.73	1.15 - 2.60	23.17	13.89 - 38.66	0.9779
24	1.18	0.63 - 2.22	7.75	5.20 - 11.54	0.9637
48	0.70	0.34 - 1.44	5.12	3.55 - 7.39	0.9833
72	0.40	0.04 - 4.26	1.69	0.46 - 6.16	0.8166

3.3 Efficacy Study of Tobacco Extracts in Controlling Stable Flies in Beef Cattle in Field Conditions

Field trials were conducted under average meteorological conditions of 26°C temperature, 60-65% relative humidity, no rainfall, with southeast winds at 10-30 km/h. No adverse effects were observed in any cattle during the entire experimental period, including skin irritation, behavioral changes, or alterations in feeding patterns following application of either cypermethrin or tobacco extract treatments. The results of the efficacy study of tobacco extracts in controlling stable flies in field conditions showed that the number of stable flies on the front legs of beef cattle differed with high statistical significance ($P<0.001$) between experimental treatments across all counting periods. Cypermethrin 35% (Treatment 2) showed the highest efficacy in controlling stable flies throughout the experimental period, with the lowest number of flies observed (5.75-8.00 flies/cattle). This was followed by 3% tobacco extract (Treatment 3), which showed 7.75-11.00 flies/cattle, while the control treatment (Treatment 1) had the highest number of flies (10.50-13.50 flies/cattle) (Table 3). It was observed that the efficacy of both substances tended to decrease over time, especially the tobacco extract, where the number of flies increased from 7.75 at 24 hours to 8.00 at 48 hours, 8.75 at 72 hours, 9.75 at 96 hours, and 11.00 at 120 hours, becoming increasingly similar to the control group (13.50 flies). This indicates that the residual effect of tobacco extract has a shorter duration than cypermethrin, likely because nicotine in tobacco is a natural substance that degrades faster than synthetic chemicals (Table 4). However, safety considerations must be addressed when using tobacco extracts containing nicotine. Neonicotinoids are a group of insecticides composed of seven types of insecticides, including acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram/nithiazine, thiacloprid, and thiamethoxam, which have been developed as safer alternatives compared to other insecticide groups such as carbamates, organochlorines, and pyrethroids commonly used worldwide. Studies by Leena et al. [29] reported that acetamiprid and imidacloprid cause reproductive toxicity, genotoxicity, neurotoxicity, and hepatotoxicity in newborn mice, but in adult mammals, toxicity levels are low. The effects of imidacloprid on the nervous system of adult mice require doses of 150 and 300 mg/kg body weight [30]. Regarding effects on humans, studies of impacts on farmers in Greece by extracting genomic DNA from blood samples of farmers and measuring levels of 8-hydroxydeoxy-guanosine (8-OHdG) found that farmers who used neonicotinoid compounds for extended periods showed increased levels of imidacloprid in their blood samples [31]. Furthermore, Loser et al. [32] studied certain metabolites of imidacloprid in nicotine-like forms, studying metabolites desnitro-imidacloprid and imidacloprid-olefin, and found low-level effects on human nervous systems. Farm workers should use appropriate personal protective equipment when handling tobacco extracts, including gloves, masks, and protective clothing to minimize skin contact and inhalation exposure. Regarding withdrawal periods for treated cattle, while nicotine is a naturally occurring compound that degrades relatively quickly in biological systems, further research is needed to establish appropriate withdrawal periods for meat and milk from cattle treated with tobacco extracts. The rapid degradation of nicotine compared to synthetic insecticides suggests that withdrawal periods may be shorter than those required for conventional chemical treatments. Still, comprehensive residue studies must be conducted to ensure food safety and comply with regulatory requirements. The establishment of maximum residue limits (MRLs) for nicotine in beef and dairy products would be essential for the commercial application of tobacco extract treatments. From an organic farming perspective, nicotine cannot be used in organic crop production because nicotine contains compounds that are classified as insecticides. Although nicotine is extracted from tobacco leaves, which are plants, organic farming certification has restrictions regarding

chemical residues in cultivated crops or in insect control applications. This limitation significantly restricts the use of tobacco extracts in certified organic livestock operations, despite their botanical origin [33]. Comparing the efficacy at 120 hours (5 days) after spraying, cypermethrin 35% could still reduce the number of flies by approximately 40.7% (compared to the control group), while 3% tobacco extract could reduce the number of flies by only 18.5%. This information is practically important for farmers in planning the frequency of spraying, especially when using tobacco extract, which may require more frequent application than cypermethrin. From an economic perspective, preliminary cost analysis reveals important considerations for practical implementation. Commercial cypermethrin 35% costs approximately 80 baht per 100 mL, while tobacco extract can be produced locally from waste tobacco products at significantly lower material costs. Although tobacco extract requires more frequent application due to its shorter residual effect, using agricultural waste tobacco materials offers substantial economic benefits, particularly for small-scale farmers who can produce extracts on-farm. The economic viability of tobacco extract becomes more attractive when considering the dual benefits of waste utilization and reduced dependency on imported synthetic chemicals, despite the increased labor costs associated with more frequent applications. However, a comprehensive economic analysis should include production costs, application frequency, labor requirements, and potential yield benefits from improved animal welfare to provide farmers with complete cost-benefit information for decision-making.

Furthermore, when comparing this study's results with research by Mullens et al. [15], the number of stable flies (*S. calcitrans* L.) in this study was considerably higher. The control group had an average of 10.50-13.50 flies per front leg, while Mullens et al. [15] reported a maximum of only 3.0-3.5 flies per leg (in late May). This difference may be due to geographical and climatic factors in Thailand, which have higher humidity and temperature (average 26°C), conditions conducive to fly development. This aligns with research by Mullens & Peterson [14], which indicated that March rainfall strongly correlates with stable fly density ($R^2 = 0.726$), as moisture promotes egg-laying and larval development. Additionally, the semi-confined farming system in the study area may have provided more breeding sites for flies compared to more strictly managed farms.

Table 4. Average number of stable flies on the front legs of cattle after spraying test substances.

Treatment	Hours after spraying				
	24	**48**	**72**	**96**	**120**
Treatment 1: Distilled water (control)	10.50[a]	11.00[a]	13.00[a]	13.00[a]	13.50[a]
Treatment 2: 35% Cypermethrin	6.00[c]	5.75[c]	6.50[c]	8.00[c]	8.00[c]
Treatment 3: 3% Tobacco extract	7.75[b]	8.00[b]	8.75[b]	9.75[b]	11.00[b]
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

[a-c] Means in the same column with different superscripts are statistically significantly different.

Comparison with other botanical insecticides reveals the diversity of plant-based pest control options. Research has been conducted on the efficacy of various plant extracts in controlling Stomoxys calcitrans through contact methods, demonstrating the diversity of plant resources with potential for development as natural insect control agents. For tobacco leaf extract use, studies found that nicotine concentrations of 25.2 mg/L could achieve LC_{50} mortality in stable flies within 24 hours after testing. Comparative efficacy studies of various plant extracts found that rosalva (from rose flowers) had the highest efficacy with LD_{50} values of 13.10 μg/cm² and LC_{90} values of 18.54 μg/cm², followed by geranyl acetone (from cardamom, orange, and petitgrain essential oils) with LD_{50} values of 25.20 μg/cm² and LC_{90} values of 34.97 μg/cm². Citronellol (from eucalyptus) had LD_{50} values of 35.69 μg/cm² and LC_{90} values of 50.10 μg/cm². Black pepper (*Piper nigrum* L.) extract had the lowest efficacy in this group with LD_{50} values of 78.37 μg/cm² and LC_{90} values of 55.62 μg/cm² when tested with 24-hour exposure periods [34-36]. These efficacy differences reflect the diversity of active compounds in each plant species, which is valuable information for selecting and developing the most effective natural insect control agents for stable fly management in livestock systems.

Additionally, recent studies have demonstrated the effectiveness of botanical insecticides in livestock systems. Plant extracts have been used to control livestock insects such as flies, ticks, and mites. The use of bioactive compounds extracted from plants has been studied as insecticides in livestock systems to be effective against insect populations resistant to drugs and have relatively low environmental impacts [37]. The biodiversity of plant extracts shows toxicity to insects in livestock systems, particularly regarding modes of

action through contact or ingestion, with different mechanisms of action for insects in the order Diptera [38]. The efficacy of essential oils from lettuce (*Lactuca sativa*), chamomile (*Matricaria chamomilla*), anise (*Pimpinella anisum*), and rosemary (*Rosmarinus officinalis*) against Lucilia sericata has been evaluated, showing LD_{50} values of 0.57, 0.85, 2.74, and 6.77%, respectively [39].

The potential for resistance development in stable fly populations is an important consideration for long-term management strategies. While botanical insecticides like tobacco extract may have multiple modes of action due to their complex chemical composition, repeated use of any single control method can lead to selection pressure and potential resistance development. The diverse alkaloid profile in tobacco extracts, including nicotine, nornicotine, and anabasine, may provide some protection against rapid resistance development compared to synthetic insecticides with single active ingredients. However, monitoring programs should be established to detect early signs of reduced efficacy in treated fly populations. Rotation between different botanical extracts and integration with other control methods would help delay resistance development and maintain the effectiveness of these natural alternatives.

Regarding fly-repelling behaviors, tail flicking and skin twitching showed highly significant differences between treatments ($P<0.001$) across all time periods. Cattle in the cypermethrin 35% group exhibited these behaviors least frequently, followed by the tobacco extract group, while the control group showed these behaviors most frequently. For head swinging behavior, significant differences were found across all time periods, with cattle in the cypermethrin 35% group showing this behavior least often. No statistical differences were found between treatments in foot stamping behavior ($P>0.05$). However, at 96 and 120 hours after spraying, observational trends indicated declining efficacy of tobacco extract, with fly counts increasing from 8.75 flies/cattle at 72 hours to 9.75 flies/cattle at 96 hours and 11.00 flies/cattle at 120 hours (Table 4). Correspondingly, fly-repelling behaviors showed increasing trends, with tail flicking behavior rising from 11.00 times at 72 hours to 11.25 times at 96 hours and 11.75 times at 120 hours, though tobacco extract treatment remained significantly better than the control treatment at all time points ($P<0.001$) (Table 5).

Considering trends over time, the frequency of fly-repelling behaviors in both test substance groups showed a slight increasing trend, corresponding with the increasing number of flies observed on the front legs of cattle after spraying. This demonstrates that the efficacy of the test substances decreased over time. The results of fly-repelling behavior in this research align with Mullens et al. [15], who found that tail flicking and skin twitching occurred more frequently than foot stamping and head swinging, which is consistent with natural cattle behavior. However, Mullens et al. [15] found that foot stamping was most effective in repelling flies, with cattle that stamped more frequently tending to have fewer flies. An interesting observation in Mullens et al. [15] was the adaptation of cattle to pain from fly bites (habituation to pain), with the ratio of foot stamping and head swinging to fly numbers decreasing significantly over time. This may explain why this study found no statistical differences in foot-stamping behavior between experimental groups, possibly because cattle on the farm had already adapted to the disturbance of numerous flies. Additionally, Mullens et al. [15] found that older cattle tended to have more flies and stamped less frequently than younger cattle.

This temporal reduction in efficacy may be influenced not only by the degradation of active compounds but also by environmental factors, such as ambient temperature and relative humidity, which are known to affect stable fly activity and cattle response [40]. These behaviors are primary reflexive responses to biting flies and tend to increase with higher fly pressure. In support of this, Rencinova et al. [41] noted that fly activity increased in association with dry, hot conditions, resulting in more pronounced behavioral responses such as tail flicking and skin twitching in dairy cattle. Furthermore, ElAshmawy et al. [40] observed that environmental conditions and management factors, such as the presence of trees around cattle pens and specific feed components in the TMR, influenced stable fly activity, which in turn may alter the expression of fly-repelling behaviors like foot stamping. Taken together, the findings from this study and those of Gerry et al. [42] highlight the complexity of behavioral responses to stable flies, which are modulated not only by the efficacy of repellent substances but also by broader environmental and management contexts that shape cattle behavior over time.

Table 5. Average frequency of fly-repelling behaviors after spraying test substances.

Treatment	Hours after spraying				
	24	48	72	96	120
Tail flicking					
Treatment 1: Distilled water (control)	14.50[a]	14.50[a]	14.50[a]	14.50[a]	15.00[a]
Treatment 2: 35% Cypermethrin	8.00[c]	8.00[c]	7.75[c]	8.50[c]	9.25[c]
Treatment 3: 3% Tobacco extract	11.00[b]	10.50[b]	11.00[b]	11.25[b]	11.75[b]
P-value	<0.001	<0.001	<0.001	<0.001	<0.001
Skin twitching					
Treatment 1: Distilled water (control)	11.50[a]	11.50[a]	11.50[a]	11.50[a]	12.00[a]
Treatment 2: 35% Cypermethrin	7.50[b]	7.50[b]	8.00[b]	8.00[b]	8.50[c]
Treatment 3: 3% Tobacco extract	9.00[b]	8.75[b]	9.25[b]	9.25[b]	10.00[b]
P-value	<0.001	<0.001	0.003	0.001	<0.001
Foot stamping					
Treatment 1: Distilled water (control)	2.50	2.50	2.50	2.00	2.50
Treatment 2: 35% Cypermethrin	1.25	1.50	1.50	1.50	1.50
Treatment 3: 3% Tobacco extract	1.75	2.25	2.50	2.25	2.50
P-value	0.241	0.73	0.57	0.532	0.57
Head swinging					
Treatment 1: Distilled water (control)	5.00[b]	5.00[a]	4.75[a]	5.00[a]	5.50[a]
Treatment 2: 35% Cypermethrin	2.00[b]	2.50[b]	2.50[b]	2.50[b]	2.50[c]
Treatment 3: 3% Tobacco extract	3.50[ab]	2.75[b]	4.00[a]	4.00[a]	4.50[b]
P-value	0.007	0.035	0.009	0.003	<0.001

[a-c] Means in the same column with different superscripts are statistically significantly different.

The integration of tobacco extract with other IPM strategies represents the most sustainable approach to stable fly management. Integrated Pest Management (IPM) is a practical approach for controlling *S. calcitrans* by combining biological, physical, and chemical control methods. However, managing this insect species is challenging due to its behavioral characteristics of intermittent blood feeding rather than permanent host residence. When considering the effectiveness of individual control methods, no single method can provide complete control results. Therefore, using multiple methods in combination is essential.

Studies by Gonzalez et al. [43] during 2022-2023 on stable fly management in horses using natural enemies combined with herbal plant extracts found that insect populations tended to decrease and horse health improved. However, the study could not definitively conclude that the population decline was solely due to IPM implementation, as other factors might affect changes in this livestock fly population, particularly climatic factors including temperature, rainfall, and sunlight that differed between study periods. This data aligns with research by Taylor et al. [44], which found that climate change affects stable fly flight behavior, reproduction, and distribution, with population density varying seasonally due to temperature and rainfall changes. Therefore, evaluating IPM effectiveness must consider the influence of these environmental factors concurrently.

4. Conclusions

Tobacco leaf fermentation at 48 hours yielded the highest nicotine content, with a significant decline in nicotine content as fermentation time increased. Laboratory test results showed that the efficacy of tobacco extract in eliminating stable flies correlated with concentration and exposure time. At 20% concentration, the extract showed maximum effectiveness (100% mortality at 48 hours), while the 3% concentration extract caused 50% fly mortality within 12 hours (LC_{50}) and was therefore selected for field testing. Field experiment results confirmed that 3% tobacco extract significantly reduced stable fly numbers and decreased fly-repelling behaviors in cattle compared to the control group. Nicotine extract from tobacco thus represents a potential alternative for controlling stable flies, especially in environmentally friendly production systems.

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