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Development of Concentrated Emulsion containing *Nicotiana tabacum* Extract for Use as Pesticide

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ABSTRACT

Using herbs as an insecticide or pesticide is well known in traditional agriculture. They are biodegradable and also friendly to environment. However, developing of commercial product from herbal plants was limited due to degradation of active ingredients, the variation of active content and there is no standard procedure for quality control. In this research, crude extracts of tobacco leaves (*Nicotiana tabacum* Linn., Solanaceae) containing nicotine as an active ingredient were studied for developing as concentrated emulsion preparation. Crude tobacco extracts from 95% ethanol were obtained as a brown syrupy mass with strong odors and 19.55% yield. One of the active ingredients, nicotine was selected to be used as a marker in suitable high performance liquid chromatography (HPLC) system in this study. The tobacco extract was stable under acid, base and heat conditions. Therefore, it was selected for further development as a concentrated emulsion formulation. The concentrated emulsion of tobacco extract composed of 10% w/w nicotine was prepared by combining fixed oil (palm oil), emulsifiers (Tween and Span), giving a more physically stable product. Under room temperature and 70 % RH for 6 month, the overall of % amount of nicotine in the product still remained in acceptable level. In the next step, the product was studied in the field with various dilution ratios of water to find out a suitable concentration of product using in agriculture field. It showed that all of the exhausted died and the plants trials are still green and not burned when the dilution is 100 time of its product.

INTRODUCTION

Pests and insects are main problem of agriculture that damage many crop plants. Various methods have been used to protect the crops from these natural enemies. Although using of pesticides is recognized as the most widely used method to solve this problem; however the health risks and environmental effects from their uses should be concerned (Feol *et al*., 2011). In traditional agriculture, the herbal pesticides are of special interest because they are biodegradable and friendly to environment (Rahman *et al*., 1999). The disadvantage of traditional agriculture is the difficulty to control product efficacy due to the variation of active constituents emerges from species differences, places of plantation and seasons of harvesting as well as no standard protocol for their quality control available.

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It has been known that tobacco leaves (*Nicotiana tabacum* Linn., Solanaceae) have been used as insecticide and pesticide for long time (Abdul-ghany *et al*., 2011). Tobacco extract is called ecological or green pesticide because of deriving from organic sources and environmentally friendly. However, tobacco extracts contains ingredients which could easily evaporate and have unpleasant appearance as brown syrupy mass and strong odors. Nowadays, there are many commercial products of tobacco extract which used technology of high-priced, complicated to prepare absorption material. This study aims to develop formulation of the tobacco extract as concentrated emulsion which shows good physicochemical characteristic and good effect in order to scale up for commercial purpose.

MATERIALS AND METHODS

Plant materials

Nicotiana tabacum used in this study were collected from Hat Yai district, Songkhla Province in April 2008.

The voucher specimen (number: NT51001) was kept by the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai campus, Songkhla, Thailand.

Chemical Materials

The reference standard of nicotine hydrogen tartrate was purchased from Merck, Germany (purity 98.0%). Acetonitrile, (HPLC grade), methanol (AR grade), acetone, *n*-hexane were purchased from Labscan Asia Co., Ltd., Bangkok, Thailand. Sulfuric acid, phosphoric acid, chloroform ($CHCl_3$), 95% ethanol (EtOH) and ethyl acetate (EtOAc) were purchased from Merck, Germany. Palm oil, rice bran oil, soy bean oil, Tween 20, Tween 80 and Span 80 were commercial grade and purchased from S. Tong Chemicals Co., Ltd., Thailand.

METHODS

Preparation of *Nicotiana tabacum* extract from tobacco leaves

Dried tobacco leaves (about 40 g) were cut in pieces and then macerated in 100 ml of five different solvents such as *n*-hexane, chloroform, ethyl acetate, methanol and 95% ethanol for 3 days. Next step, each filtrate was evaporated by rotary evaporator. The maceration was repeated 3 times to get total crude extract. The tobacco extract were analyzed by thin layer chromatography (TLC) using nicotine standard for comparison. %Yield of tobacco extract was calculated in order to find out the effective solvent of extraction.

Determination of nicotine content in the tobacco extract

The amount of nicotine in the tobacco extract was analyzed by using high performance liquid chromatography (Agilent® 1100 series, Palo Alto, CA). The column was Hypersil® BDS C18, 250x4.6 mm, 5 µm. The mobile phases were 0.14% v/v triethylamine in water: acetonitrile (70:30 v/v), flow rate at 1.0 ml/min, UV Detector was set at 260 nm, injection volume was 10 µl (USP27). Firstly, the standard solutions were prepared to have concentrations equivalent to 3.6, 18.0, 36.0, 72.0 and 180.0 µg/ml of nicotine using methanol as a solvent. After that, these standard solutions were taken to analyze by HPLC to construct standard curve between actual concentrations of nicotine and peak areas. After that, the solution of the tobacco extracts was dissolved in methanol to give the solution having concentration of 112, 120 and 160 µg/ml. Then, these samples were taken to analyze by using HPLC and calculated to determine the amount of nicotine in the tobacco extracts by using nicotine standard curve.

Stability indicating assay

The condition of HPLC system which is modified from assay method of nicotine transdermal system of USP27 was evaluated for the suitability for determination of nicotine clear separately from other compounds in the extracts or degraded products under accelerated condition. The accelerated condition composed of three factors such as acid, base and heat which affect stability of the tobacco leaves extract. The method of acceleration by acid was prepared as follow. The tobacco extract 25 mg was dissolved in 1.5 ml methanol and 1.5 ml of 5% hydrochloric acid and was kept at 25±1°C until 24 hours, after that 1.5 ml of 5% sodium hydroxide was used to adjust pH to neutral. A suspension of extract was filtered through membrane porous size 0.45 micron and the filtrate was determined by HPLC system. In the other hand, acceleration by base was prepared in the same manner using 5% sodium hydroxide solution as base and 5% hydrochloric acid as neutralizing agent.

In heat accelerated degradation experiment, the tobacco extract 25 mg was dissolved in 1.5 ml methanol and 1.5 ml of distilled water at 80±1°C until 24 hours, after that a suspension of extract was filtered through membrane porous size 0.45 micron and determined by HPLC system.

Stability study of the tobacco extract

The tobacco extract was weighed 2 g in each nine closed bottle glasses. Each three bottles were kept in desiccators with controlling condition at 70% RH and 25±1°C, 70%RH and 45±1°C and 70% RH and 70±1°C, respectively. The sampling periods to analyze the nicotine content by HPLC were 7, 14, 21, 42, 63 days, respectively.

Pre-formulation study of the concentrated emulsion of the tobacco extract

Solubility study of the tobacco extract in solvents

An excess amount of the tobacco extract was weighed and added in the solvents. The selected solvents which were used for solubility study in order to find out the suitable solvent system of a concentrated emulsion formulation composed of distilled water, ethanol, methanol, 5%, 10% and 15% w/w of Tween 80 in water. All of the solvent systems containing excess extracts were shaken at 25±1°C until 24 hours and filtered through membrane 0.45 micron before analyzing by HPLC method.

Preparation of the concentrated emulsion containing the tobacco extract

The concentrated emulsion formulations were prepared by emulsification method without process of heating. The concentrated emulsion of tobacco extract composed of 10% w/w nicotine was prepared by combining fixed oil (rice bran oil, palm oil, soy bean oil), emulsifiers (Tween and Span), giving a more physically stable product. All of compounds of the concentrated emulsion were homogeneously mixed together by homogenizer.

Investigation of physicochemical characteristics of the concentrated emulsion containing the tobacco extract

In this study, the observed characteristics of the formulations were determined as color and homogeneity with naked eyes. The acid-base values were evaluated by pH meter (Orion 410A, American Instrument Exchange Inc., USA). Viscosity values were determined by a Brookfield viscometer fitted with RV-7 spindle and 5 rpm spindle speed (Brookfield

Engineering Laboratories Inc., USA). All measurements were performed in triplicate at room temperature.

Stability study of the concentrated emulsion containing the tobacco extract formulations

Stability studies were conducted under freeze-thaw conditions. In each cycle, each sample was kept at 4°C for 24 h and at 45°C for 24 h. The sample was then evaluated for its stability after 6 cycles. Furthermore, the stability studies also conducted under room temperature (25±1°C) for 1, 3 and 6 months, respectively. Changes of physical appearance, pH, viscosity and content of the nicotine were determined.

Study of dilution ratios of the concentrated emulsion as pesticide

The selected concentrated emulsion formulation was used to test effect of the tobacco extract on aphids (*Aphis glycines* Mats.) in the experiment field. The concentrated emulsion was diluted to ratio 1:10, 1:20, 1:50, 1:80 and 1:100 with distilled water in order to clarify the optimum dilution for application. These dilutions of product were used in the field and then the dead aphids and condition of crops or vegetables were observed with the period of 0, 2 and 24 hours, respectively.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's multiple comparison test and t-test were used to investigate the statistical significance of differences and a *P* value of 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Effect of solvents on extraction of tobacco leaves

The extraction of tobacco leaves from 5 solvents such as *n*-hexane, chloroform, ethyl acetate, methanol and 95% ethanol gave a different percent yield of extraction which shown in Table 1.

Table. 1: Percent yield of extraction from different solvents.

Solvent	Weight (g)		% Yield
	Tobacco leaves	Tobacco extract	
n-hexane	40.0	1.18	2.95
chloroform	43.4	4.22	9.72
ethyl acetate	40.6	4.68	11.53
methanol	40.1	7.18	17.91
95% ethanol	40.0	7.26	18.15

The tobacco extract which used 95% ethanol as a solvent gave a highest percent yield of extraction. Therefore, 95% ethanol was chosen for reprocess of high scale of extraction because of safety and high productivity.

However, the cost of extract might be more expensive than other solvents. A scale up process of extraction by 95% ethanol was reprocessed. Then the dry tobacco leaves were weighed 5 kg and macerated in 12.5 kg 95% ethanol for 3 days. The percent yield of extraction of a scale up process was 19.55% and the tobacco extract appeared as brown syrupy mass with strong odors as shown in Figure 1. TLC fingerprint of the tobacco extract was determined to compare with standard of nicotine. The result of TLC profile revealed that the tobacco extract composed of not only nicotine but also other compounds with variety of polarity.

Fig. 1: The tobacco leaves macerated with 95% ethanol in round bottom flask (Left) and an appearance of the tobacco extract (Right).

Analysis to define the amount of nicotine in the tobacco extract

A nicotine content of tobacco extract was examined by using HPLC method. The HPLC chromatogram of nicotine was shown in Figure 2A.

The result showed that the amount of nicotine in tobacco extract equals 13.47±0.66 % w/w. This concentration is higher than the concentration from the study of Casanova *et al*., 2004. In addition, Casanova reported that concentration of nicotine in the crude extract was 3.36% w/w. However, they used oleic acid as an extraction medium which different from this study and also different extraction method (an effleurage method). A chromatogram of the tobacco extract displayed a peak of nicotine at the retention time of 9.35-9.40 minutes. Furthermore, the HPLC system could resolve nicotine from other components in the tobacco extract very well.

Stability indicating assay

The HPLC condition system for determining the other compounds in the extracts or degraded products under accelerated condition such as acid, base and heat was suitable assay method to clearly separate all of compounds in the tobacco extract. The HPLC chromatograms of the tobacco extract under normal, acid, base and heat condition were depicted in Figure 2A- 2D, respectively.

The results revealed that the retention time of nicotine under all conditions were the same at 9.3 minutes while as other compounds or degrade products were stated at the retention below 9.3 minutes . However, the tobacco extract was still stable under accelerated conditions. Soloway (1976) reported about the major metabolites of nicotine which were composed of hydroxynicotine, cotinine, desmethylcotinine and hydroxycotinine. The metabolic pathway is the primary process occurring in plants, insects and human by oxidative process. In this study, the detail of degrade products or metabolite compounds were not studied.

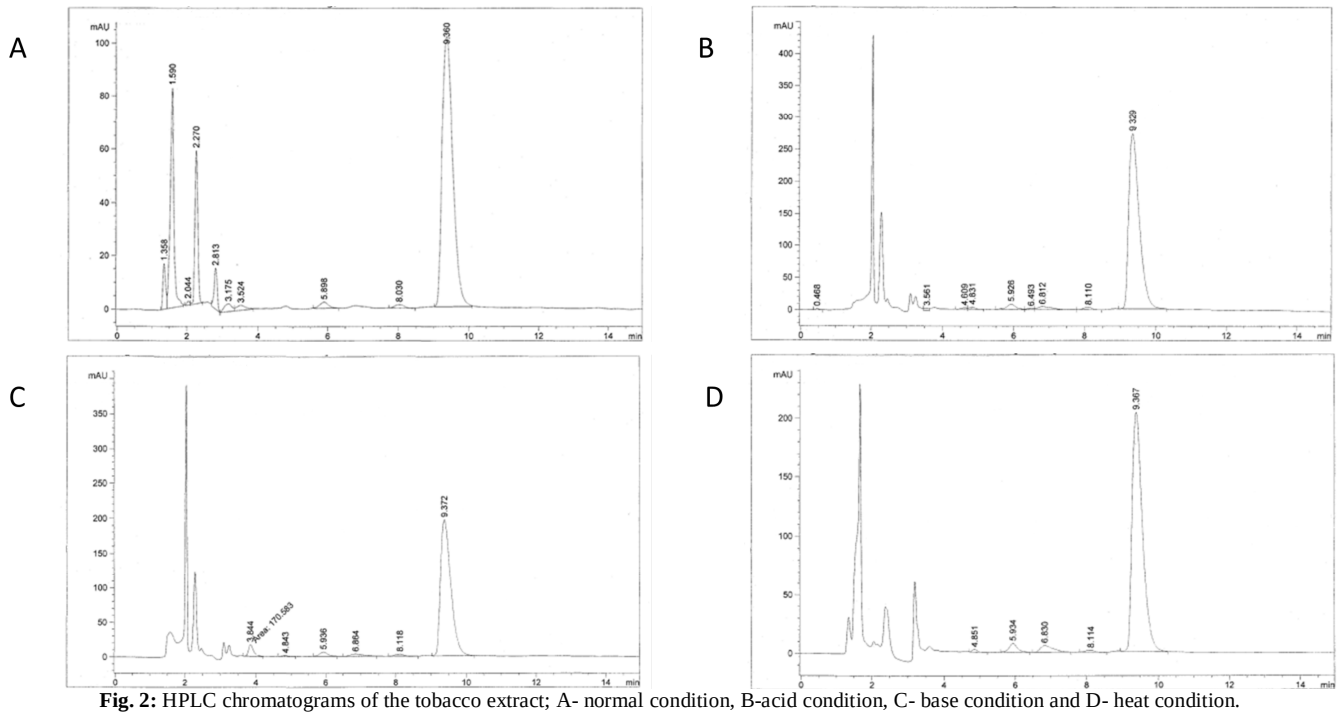

Fig. 2: HPLC chromatograms of the tobacco extract; A- normal condition, B-acid condition, C- base condition and D- heat condition.

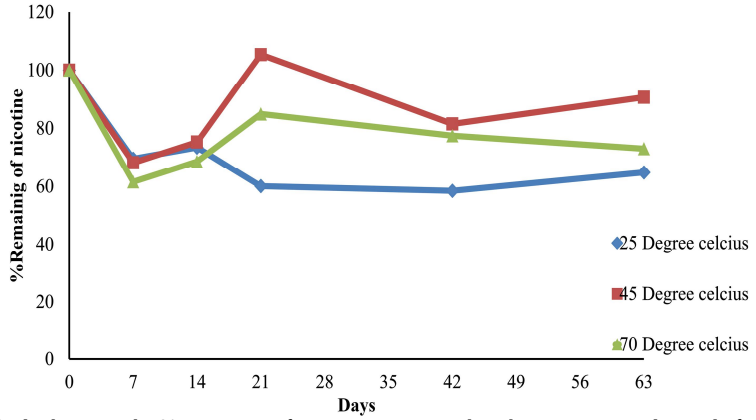

Fig. 3: The stability profiles which plot between the % remaining of nicotine content in the tobacco extract and period of time at 7, 14, 21, 42, 63 days under controlling condition at 70% RH and 25±1°C, 70%RH and 45±1°C and 70% RH and 70±1°C.

Stability of the tobacco extract

The stability profiles which plot between the % remaining of nicotine content in the tobacco extract and period of time at 7, 14, 21, 42, 63 days under controlled condition at 70% RH and 25±1°C, 70%RH and 45±1°C and 70% RH and 70±1°C are displayed in Figure 3. The stability curve showed that nicotine content decreased from the origin even though there was fluctuation of contents between the observed periods. This might due to the remaining of ethanol in the tobacco extract which disturbed the final concentration of nicotine when it evaporated under a high temperature (boiling point of ethanol is 78.4 °C). The tobacco extract had a brown syrupy mass that the solvent could not be completely removed. At two months, the content of nicotine in the tobacco extract still remained about over 65% w/w for all conditions. However, these extracts still were further used in formulation development.

Effect of solvent on solubility of pre-formulation

The solubility of the tobacco extract in various solvents which selected to develop a concentrated emulsion formulation was displayed in Table 2.

The data showed that the solubility values of various solvents were not significantly different. 15% w/w of Tween 80 gave the highest solubility of tobacco extract. Tween 80 affected solubility value of the tobacco extract, with increased amount of Tween 80, the solubility of the tobacco extract was also increased. Tween 80 is a nonionic surfactant which acts as micelle structure to solubilize the tobacco extract in water easily (Martindale, 1993). However, the concentration of Tween 80 between 10 and 15% w/w did not clearly gave different solubility, therefore the selected concentration for further formulating was 10% to save of the cost of the product.

Table. 2: Solubility of the tobacco extract in various solvents.

Solvent	Amount of the tobacco extract (mg/ml)
H_2O	32.5 ± 0.3
H_2O + 5%Tween 80	32.5 ± 0.7
H_2O + 10%Tween 80	33.5 ± 1.0
H_2O + 15%Tween 80	33.7 ± 0.6
Ethanol	33.5 ± 0.8
Methanol	34.2 ± 0.5

Effect of compound on the concentrated emulsion containing the tobacco extract

The concentrated emulsion of tobacco extract composed of 10% w/w nicotine was prepared by combining 5% w/w of fixed oil (rice bran oil, palm oil, soy bean oil) and 20 % of emulsifiers (Tween 80 and Span 80), giving a more physically stable appearance which shown in Figure 4.

As known before, nicotine and other volatile constituents in the tobacco extract could evaporate easily then the fixed oils such as rice bran oil, palm oil or soy bean oil might help to delay the rate of evaporation of this compound. Two phases of solvents between oil and water are not miscible, so Tween 80 and Span 80 were used to emulsify the two phases homogeneously. The solubility values of nicotine in 10% w/w of Tween 80 was 3% w/w. This value was lower than an amount of nicotine in the concentrated emulsion (10% w/w). Therefore, Span 80 was used as a mixture with Tween 80 to obtain suitable emulsion system for stabilizing the high amount of tobacco extract in this formulation. The cold process of emulsification was applied to avoid the heat effect on the evaporation of the tobacco extract.

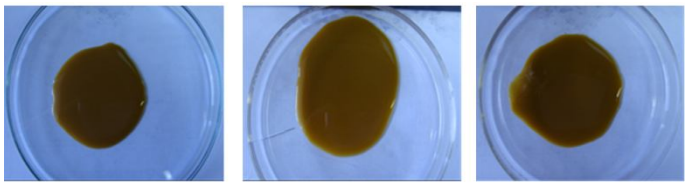

Fig. 4: The appearance of the concentrated emulsions which composed of different fixed oils; A- Rice bran oil, B- Palm oil and C- Soy bean oil.

Physicochemical characteristics of the concentrated emulsion

The observed characteristics of the formulations were determined in the aspects of color, homogeneity, pH and viscosity values shown in Table 3. The results showed that the formulation composed of palm oil as fixed oil gave the highest viscosity while as pH values were not different among three formulations. Furthermore, the color of its product was light brown which more pale color than other two formulations.

Stability of the concentrated emulsion

Stability studies under freeze-thaw conditions were determined color, homogeneity, pH and viscosity values as shown in Table 3. The formulation composed of palm oil did not show significantly change of color, pH and viscosity between before and after freeze-thaw condition when compared to other formulations, therefore, it was selected for further analyzing nicotine content under room temperature (25±1°C) for 1, 3 and 6 months, respectively. Physicochemical properties of the formulation composed of palm oil under room temperature were displayed in Table 4. At the end of period of stability study (6 months) overall appearances were not different from the freshly prepared except for much higher viscosity values. The reasons of a high viscosity value of the product without changing of volume and pH value might be a result of components in formulation. One of the suspected compounds was the palm oil, of which the lipid structure composing of glyceryl ester of fatty acid. The weak acidic pH of the formulation might stimulate ester hydrolysis and the resulting carboxylate species formed salt with the cationic nicotine and give rise to decrease of nicotine evaporation. The amount of nicotine content also had high value (93% w/w) with the acceptable range of product stability within 6 months.

Effect of the dilution ratios on the efficacy of the concentrated emulsion as pesticide

The concentrated emulsion was diluted to ratio 1:10, 1:20, 1:50, 1:80 and 1:100 with water and then tested in the field experiment (Fig 5B-5F). The emulsions of all of dilution ratios were capable to destroy aphids without crop damage. The results were shown in Figure 6A-6C with the dilution 1: 100 at time period of 0, 2 and 24 hours. Soloway, 1976 reported that the concentration of nicotine used as insecticide was 0.5-1% w/w for vegetables. In the case of inhibition oviposition behavior of *Helicoverpa armigera* moths used nicotine sulfate only 0.3% w/w (Sehgal *et al*., 2003). However Yamamoto *et al*., 1999 reported that the 50% lethal dose of nicotine by contact poison were 50 mg per kilogram of body weight for human beings. Therefore, the balance of safety and effectiveness to use this product is important. This study showed the dilution 1:100 which contained amount of nicotine about 0.1% w/w (100 mg) could destroy aphids in trial field as well with safety enough for human.

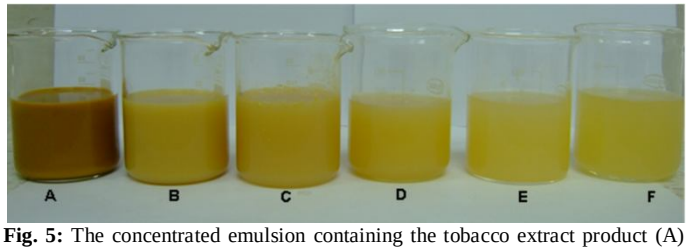

Fig. 5: The concentrated emulsion containing the tobacco extract product (A) and the various dilution ratios of the product with water; B- 1:10, C- 1:20, D- 1:50, E- 1:80 and F- 1:100.

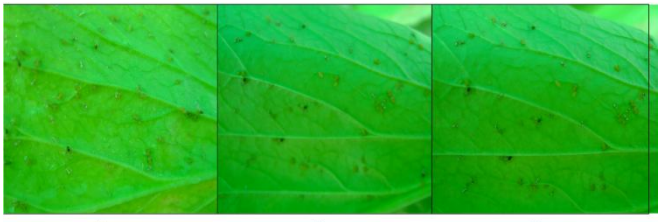

Fig. 6: The appearance of the aphids on vegetable leaf when using the dilution ratio 1:100 of the concentrate emulsion at various time periods of 0 (A), 2 (B) and 24 hours (C).

Table. 3: Physicochemical characteristics of freshly prepared and under freeze-thaw condition of the concentrated emulsion containing the tobacco extract.

Fixed oil	Freshly Prepared			Freeze-thaw condition 6 cycles		
	Color	pH	Viscosity (cps)	Color	pH	Viscosity (cps)
Rice bran	brown emulsion	5.29±0.02	135.77±0.00	brown emulsion	5.12±0.015	239.20±0.80
Palm	light brown emulsion	5.26±0.02	216.05±0.17	light brown emulsion	5.18±0.031	284.95±0.44
Soybean	brown emulsion	5.25±0.02	115.11±0.12	brown emulsion	5.06±0.06	219.80±0.42

Table. 4: Physicochemical properties of the concentrated emulsion of palm oil under room temperature at various time periods of stability study.

Month	The concentrated emulsion of palm oil under room temperature				
	Color	pH	Viscosity (cps)	% nicotine (Labeled amount)	% remaining
1	Light brown emulsion	5.29±0.03	798.93±0.48	122.56±3.41	100.00
3	Light brown emulsion	5.27±0.03	857.42±0.69	117.14± 0.31	95.57
6	Light brown emulsion	5.27±0.03	908.02±0.18	114.47±3.37	93.39

CONCLUSIONS

The concentrated emulsion of tobacco extract showed to be the effective product with physical appearance stability including a percent amount of nicotine in the product still remain in acceptable level over 6 months. The efficiency of product which studied in the field revealed that all of the aphids died and the plants trials are still green and not burn when the dilution is 100 time of its product.

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