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Abstract. Nanoemulsion is a preparation consisting of an oil phase and a water phase with particle sizes in the range of 20-200 nm. The study aimed to obtain a mixed nanoemulsion from Piper aduncum fruit and Tephrosia vogelii leaves as an active botanical insecticide against larvae of Crocidolomia pavonana. Nanoemulsions were made using a low energy method, namely spontaneous emulsification using a magnetic stirrer and then followed by a toxicity test. The nanoemulsion toxicity test was carried out singly and in a mixture on C. pavonana larvae using the deep leaf method. The results showed that at the LC50 and LC95 nanoemulsion of mixture T. vogelii and P. aduncum extract was synergistic and showed higher activity than a single nanoemulsion of T. vogelii and P. aduncum.

Key word: cabbage, mixture, nanoemulsion, synergistic, pest

1. Introduction

Previous research on mixture extract of Tephrosia vogelii and Piper aduncum had higher insecticidal activity than the single extract and was strongly synergistic. The mixed extract influenced the physiological functions of the insects tested by Crocidolomia pavonana through its antifeedant effect and disruption of food assimilation. In addition, the toxic components of P. aduncum increased the activity of detoxification enzyme of C. pavonana the cytochrome b5 and P450 (Lina et al. 2015). Botanical insecticide formulations made from T. vogelii and P. aduncum (1: 5) in the form of emulsifiable concentrate (EC) and wettable powder (WP). Several advantages of the formulations of are low persistence, soft effect against natural enemies, effective in suppressing population of C. pavonana in the field [35].

Performance improvement of the mixture formulations is needed by manipulating matter on an atomic scale called nanotechnology [36]. Some advantages of nanotechnology-based formulations are increased application surface area, facilitate the systemic activity, reduce organic solvent waste, protect active ingredients from decomposition by microorganisms and sunlight, increase solubility, extend the persistence of active ingredients, and increase the physicochemical stability of formulations. Nanoemulsion of T. vogelii extract called formula A.1 and formula A.2 was made [33]. Nanoemulsion of P. aduncum extract named formula AT.1 and BA.1 [34]. With assumption that mixture of T. vogelii dan P. aduncum has higher insecticidal activity than the activity of single extract we determine activity of mixture T. vogelii and P. aduncum nanoemulsion within cabbage pest C. pavonana.

2. Methodology

The research was conducted at the Sumatra Biota Laboratory, Faculty of Pharmacy and Insect Bioecology Laboratory, Faculty of Agriculture, Andalas University, Padang from September to
December 2019. This study used a completely randomized design (CRD) consisting of control treatment and mixed nanoemulsion treatment.

a. Extraction

The extraction of *T. vogelii* and *P. aduncum* was carried out by the maceration method using ethyl acetate as a solvent. In the first stage, *P. aduncum* fruit and *T. vogelii* leaves were each cut to a size of 1 cm and placed on a bamboo tray lined with paper. Then allowed to air dry without direct sunlight for 3 weeks. After drying, the ingredients are blended until smooth and sieved to obtain a fine form in the form of a powder. In the second stage, each powder was weighed 50 g then put into a separate Erlenmeyer flask and immersed in 500 ml of ethyl acetate solvent for 2 x 24 hours. Then the extracted liquid is filtered using a glass funnel (diameter 9 cm) which is lined with ordinary filter paper and accommodated in an Erlenmeyer flask.

The first filter results are filtered again using a glass funnel (5 cm in diameter) based on Whatman filter paper No. 41 and accommodated in a steam flask. The second filter was evaporated with a rotary evaporator at a temperature of 50°C and a pressure of 240 mbar. Ethyl acetate obtained from evaporation is used to re-soak the plant extract pulp up to 3x immersion (2 subsequent immersions for 1 x 24 hours). The extract obtained in the flask is a brown semisolid material.

b. Preparation of Insecticide Nanoemulsion

Nanoemulsion was prepared by spontaneous emulsification by modifying the technique of [17], [18], [19]. Nanoemulsion with spontaneous emulsification, namely nanoemulsion which occurs when the organic phase and the water phase are mixed. The emulsion system consists of an organic phase (extract = *T. vogelii* and *P. aduncum* (1: 3), solvent = bioethanol), and an aqueous phase (water = distilled water and surfactant = Tween 80). The organic phase was prepared by mixing the two extracts and the solvent as much as 10% of the total emulsion with a composition of 1: 1 (g / v).

The water phase was prepared by mixing 3% Tween 80 and distilled water then stirred using a magnetic stirrer for 30 minutes at room temperature. The spontaneous emulsification technique is carried out by adding the organic phase to the water phase through dropping (drop by drop) and followed by magnetic stirring for 45 minutes.

c. Insecticide Nanoemulsion Analysis

The insecticide nanoemulsion that had been prepared was then sent to the Bogor Post Harvest Center for analysis using a Particle Size Analyzer (PSA). The tool used in the PSA is the Zetasizer Nano ZS Malvern. Analytical data that can be obtained are particle size, polydispersity index, zeta potential, and particle distribution width of nanoemulsions.

d. Insecticide Nanoemulsion Toxicity Test

The nanoemulsion formula was tested by preparing it according to a predetermined concentration then the formula was dissolved in distilled water. Fresh pesticide-free broccoli leaves are cut into 4 cm x 4 cm sizes and dipped one by one in a nanoemulsion formula with a certain concentration including the control solution until evenly wet, then dry to air. After drying, each treatment leaf cut and control leaf were placed in a tissue-lined petri dish. Furthermore, 15 larvae of *C. pavonana* instar II were put in per petri dish. Leaf feed treatment was carried out for 2 x 24 hours, then the larvae were given leaf feed without treatment until they reached the IV instar. This experiment was repeated three times. Data on larval mortality were recorded daily.

e. Mixed Activity Nature Analysis

For extract mixtures whose components come from different plant species, the activity properties of the mixture were analyzed based on different working models by calculating the combination index at the LC50 and LC95 levels. The combined index (IK) at the LCx level is calculated by the following formula [20]:

\[
IK = \frac{LC_{X1}^{x}}{LC_{X2}^{x}} + \frac{LC_{X2}^{x}}{LC_{X1}^{x}} + \left( \frac{LC_{X2}^{x}}{LC_{X1}^{x}} \times \frac{LC_{X1}^{x}}{LC_{X2}^{x}} \right)
\]

\(LC_{X1}^{x}\) and \(LC_{X2}^{x}\) respectively LCx components extract 1 and 2 on separate tests; \(LC_{X1}^{x}\) (cm) and \(LC_{X2}^{x}\) (cm), respectively LC components 1 and 2 in the mixture resulting in mortality x (eg 50% and 95%). The LC value is obtained by multiplying the LCx of the mixture by the proportion of the concentration of components 1 and 2 in the mixture. The category of mixed interaction properties was adapted from Kosman and Cohen (1996) and Gisi (1996) based on the inverse co-toxicity ratio:

(1) if the CI <0.5, the components of the mixture are strong synergistic;
(2) if the CI is 0.5–0.77, the components of the mixture are weakly synergistic;
(3) if the CI> 0.77–1.43, the components of the mixture are additive;
(4) if the CI> 1.43, the components of the mixture are antagonistic.

f. Assimilation of Insect Foods

Testing of food assimilation was carried out using the residual method on the leaves of *C. pavonana* larvae by a solution of *P. aduncum* nanoemulsion with sublethal or non-lethal concentrations, namely LC25 and LC50 [1]. The aim is to determine the parameter value of the efficiency of food utilization from larvae. The pieces of broccoli leave measuring 4 cm x 4 cm were dipped in a nanoemulsion (v/v) preparation and turned around until the entire leaf surface was evenly wet, then air-dried. The cut leaves and third instar *C. pavonana* larvae were weighed to determine their weight respectively. After weighing, one larva and one piece of a leaf are put into a petri dish that has been lined with tissue and allowed to eat for 24 hours. This treatment uses 10 larvae. Observations were made 24 hours after treatment. Then the larvae, remaining feed, and feces are dried in separate ovens at 100°C until their weight is constant and weighed again. To estimate the initial dry weight, 10 larvae and 10 sample leaves (of the same size as used in the treatment) were weighed separately then immediately dried until their weight was constant and weighed again. The ratio of larvae weight or feed after and before drying is the proportion of dry weight to wet weight.

\[
\text{Dry weight (BK) = wet weight}\times (\text{sample BK}) / (\text{sample weight})
\]

g. Observation

Food assimilation data obtained consist of consumption rate (LK), larvae average weight (BRL), relative consumption rate (LKR), growth rate (LP), relative growth rate (LPR), digestibility (DC), food efficiency consumed (EMK) and the efficiency of digestible food (EMC). These parameters can be obtained on a gravimetric basis (Waldbauer, 1968):

\[
\text{LK} = \text{BK of initial feed-BK of final feed}
\]
\[
\text{BRL} = (\text{early larvae BK + late larvae BK}) / 2
\]
\[
\text{LKR} = (\text{consumption rate}) / (\text{weight average of larvae})
\]
\[
\text{LP} = (\text{DM late larvae-early larvae BK}) / (\text{feeding period})
\]
\[
\text{LPR} = (\text{Growth rate}) / (\text{Average weight of larvae})
\]
\[
\text{DC} = (\text{Waste consumption rate-BK}) / (\text{Consumption rate}) \times 100\%
\]
\[
\text{EMK} = (\text{DM of late larvae-DM of early larvae}) / (\text{rate of consumption}) \times 100\%
\]
\[
\text{EMC} = (\text{late larvae DM-early larvae}) / (\text{Dung-BK consumption rate}) \times 100\%
\]

h. The Effect of *T. vogelii*: *P. aduncum* (1: 5) Mixed Extract on Food Assimilation by *C. pavonana* Larvae

The mixed extract of *T. vogelii*: *P. aduncum* (1: 5) was tested by the leaf residue method against 3rd instar *C. pavonana* larvae at the concentration of LC25 and LC50 to determine the effect of the mixed extract on the efficiency of food assimilation. The instar larvae used in the experiment were weighed one by one. The weighed larvae are directly put into a petri dish, which contains leaves with the size of 4 cm x 4 cm which has been treated (LC25 and LC50) and the weight has been known. This treatment used 15 3rd instar *C. pavonana* larvae. Observations were made 48 hours after treatment, then the test larvae, the remaining feed, and their feces were dried in an oven separately at 100°C until their weight was constant. To estimate the initial dry weight, 10 larvae and 10 leaf samples (of the same size as used in the treatment) were weighed separately then immediately dried to constant weight and weighed again. The ratio of larvae weight or feed after and before drying is the proportion of dry weight to wet weight.

The data obtained were used to determine the value of the food utilization efficiency parameter. The parameters measured in this experiment are consumption rate (LK), relative consumption rate (LKR), growth rate (LP), relative growth rate (LPR), digestibility (DC), the conversion efficiency of food consumed (EMK), and conversion efficiency. digestible food (EMC), which is calculated on a gravimetric basis (Waldbauer 1968). The data for each parameter were processed by means of variance and the comparison of mean values between doses was carried out by using the Duncan test at the 5% real level (Steel and Torrie 1993).
3. Results and Discussion

The mixed nanoemulsion was tested for the toxicity of *C. pavonana* larvae with 6 types of concentrations along with control. Data on mortality and duration of larval development can be seen in Table 1:

**Table 1. Mortality and duration of development of Crocidolomia pavonana larvae after mixed nanoemulsion treatment**

| Concentration (%) | Mortality (%) ± SD | Duration of larval development (days) | Instar II – III ± SD | Instar II – IV ± SD |
|-------------------|--------------------|--------------------------------------|----------------------|----------------------|
| 0.15              | 90.66 ± 0.52       | a                                    | 4.42 ± 1.40          | 7.57 ± 1.92          |
| 0.075             | 58.66 ± 1.69       | b                                    | 4.67 ± 1.46          | 7.22 ± 1.97          |
| 0.375             | 46.66 ± 2.71       | b                                    | 4.00 ± 1.61          | 7.10 ± 2.30          |
| 0.018             | 18.66 ± 1.14       | c                                    | 3.29 ± 0.34          | 5.54 ± 0.49          |
| 0.009             | 12.00 ± 1.37       | c                                    | 3.24 ± 0.29          | 5.78 ± 0.33          |
| 0.00 (control)    | 0.00 ± 0.00        | d                                    | 2.52 ± 0.06          | 4.89 ± 0.10          |

* The numbers followed by the same letter in the same column were not significantly different according to the 5% LSD follow-up test; SD = standard deviation

Based on the results of research on botanical insecticides made from a mixture of *T. vogelii* and *P. aduncum* extracts, it can be seen that there is an increase in mortality of treated larvae along with increasing concentration levels. Then in the observation of larval development time, there was no effect between the level of nanoemulsion concentration on the duration of development of *C. pavonana* larvae, both at instar II - III and instar II - IV. However, in general the results indicate an extended survival period of larval development when compared to controls. The observation results can be seen in Table 2. The duration of larval development at a concentration of 0.00% (control) was 2.52 days for II-III instars and 4.89 days for II-IV instars. Then the development time of the mixed nanoemulsion treatment larvae is 3-4 days for instar II-III and 5-7 days for instar II-IV.

*P. aduncum* kills the test insects by acting as a neurotoxin. Piperamide compounds from the Piperaceae family (guininsin and pipericides) work as neurotoxins that inhibit the flow of nerve impulses to the axons causing paralysis [21], [22]. The Piperaceae family is known to have synergistic properties when mixed with other extracts. This is due to the presence of lignans containing methylenedioxyphenyl groups which can inhibit the activity of cytochrome P450 enzymes and reduce the toxicity of foreign compounds including insecticides [11], [12]. According to [13] dilapiol derived from *P. aduncum* can inhibit the activity of the enzyme cytochrome P450 in microsome preparations from the digestive tract cells of corn stem borer *O. nubilalis*.

The combination of the mixed extract works by means of facilitation, namely, as explained above, the active ingredient of *P. aduncum* inhibits the activity of enzymes that break down toxic compounds in the insect body, as a result the active ingredient of *T. vogelii* does not decompose properly so that it can enter the target and work optimally. Therefore, *P. aduncum* extract containing dilapiol has the potential to be synergistic when mixed with other plant extracts. It can be seen from Table 1 that the proportion of the concentration of *P. aduncum* is greater than that of *T. vogelii*, which is 3 times more likely to cause a greater inhibition of the PSMO enzyme activity so that the active compound *T. vogelii* can avoid breakdown by the enzyme and can continue to work. attack the target.

*Tephrosia vogelii* is known to contain rotenone compounds that are insecticidal [9], [10], [23]. Rotenon has insecticidal activity against various types of insects as a stomach poison and contacts poison [24], [25]. This disturbance occurs due to the rotenone compound contained in the botanical insecticide nanoemulsion made from *T. vogelii*. Rotenone is known to cause paralysis of the muscle system and tissue system that works when larvae digest food. [26] explained that rotenone is a cellular respiration poison that inhibits the transfer of electrons between NADH dehydrogenase and coenzyme Q in complex I of the electron transport chain in the mitochondria. Rotenone blocks the transfer of electrons from Fe-S to the ubiquinone coenzyme, thereby inhibiting the process of cellular respiration and decreasing ATP production. The inhibition of the cellular respiration process causes the production of ATP to decrease so that the cells lack energy which in turn can cause paralysis of various muscle systems or other tissues and eventually the insects die.
Nanoemulsion with this mixture, apart from being able to kill insects with a high mortality value, also has other advantages, namely having a different way of working so that it can delay the occurrence of pest resistance [27], [28]. The ability of insects to form a defense system against several different compounds at once is more difficult than against a single compound. Insects do not easily become resistant to plant extracts with some active ingredients. Many plant compounds act differently from the synthetic insecticides commonly used today, so the possibility of cross-resistance occurring is quite small [29]. In addition, mixing several botanical insecticides can also make plant material preparations efficient, as said by [30] that the use of a synergistic botanical insecticide mixture can reduce the amount of use of raw materials compared to botanical insecticides containing a single extract, so as to overcome dependencies/limitations. The raw material for botanical insecticides at the farm level because plant sources of botanical insecticides are not always abundant in an area. Botanical insecticides in the mixed form are also more economical [31], [28].

The mixing of several active plant compounds can provide effects such as synergistic, antagonistic, or additive. In a plant extract, in addition to some of the main active compounds, there are usually many other compounds that are less active, but its existence can increase the overall extract activity (synergy) [29]. Several researchers have proven the effectiveness of a mixture of two types of plant extracts to be used as a botanical insecticide. For example, the mixture of *T. vogelii* and *P. cunea* (5:9) leaf extracts was synergistic against *C. pavonana* larvae, both at the LC50 and LC95 levels [32].

Probit regression analysis was used to determine the relationship between the concentration of a botanical insecticide nanoemulsion made from a mixture of *T. vogelii* and *P. aduncum* extracts on the mortality of *C. pavonana* larvae can be seen in Table 2. Probit analysis produces the regression slope values (b value), LC50 and LC95, as follows:

| Nanoemulsion | Value ± SE | LC50 (%) | LC95 (%) | IK value at level |
|--------------|-----------|----------|----------|------------------|
| A mixture of *T. vogelii* and *P. aduncum* | 2.00 ± 0.44 0.04 | 0.3 | 0.48 | SK 0.66 SL |

* b = slope of the regression; SE = standard error; IK: combined index; SK: strong synergy; SL: weak synergy

Based on the results of the probit analysis, it is known that the b value of mixed nanoemulsion is 2.00. According to [23], adding a certain amount of concentration to a nanoemulsion treatment with a high b value would kill the test insects more than a nanoemulsion with a lower b value. Then to kill the larvae of *C. pavonana* as much as 95% required 0.3% nanoemulsion mixture of *T. vogelii* and *P. aduncum*. The mixed nanoemulsion LC95 value was lower than the LC95 value of the two single nanoemulsion treatments separately. [18] stated that to kill *C. pavonana* larvae using a single botanical insecticide nanoemulsion made from *T. vogelii* required a concentration of 1.29%. In addition, [19] also argued that to kill *C. pavonana* larvae using a single botanical insecticide nanoemulsion made from *P. aduncum* required a concentration of 0.85%. The nanoemulsion mixture of *T. vogelii* and *P. aduncum* is synergistic as a botanical insecticide. However, both of them have a combination index value with different synergistic properties, which are weak at the LC95 level, while at the LC50 level the combination is strongly synergistic. These data prove the role of the active compounds of *P. aduncum* and *T. vogelii* together to form a synergy that can kill the target pests better than singly.

The strong synergistic indication of mixed nanoemulsion was proven by the higher mortality value of mixed nanoemulsion treatment in killing *C. pavonana* larvae compared to the single/separate nanoemulsion treatment. The same thing was also explained by [32], the use of a mixture of botanical insecticides that are synergistic in nature can increase the efficiency of the application because mixed insecticides are used at a lower dose compared to the dose of each component separately. The use of
botanical insecticide mixtures at lower doses can also reduce side effects on non-target organisms and the environment. [23] added that the mixed extract activity was more active in killing *C. pavonana* larvae than the single extract. Mixed combination index analysis of 13 mixed extract combinations of *B. javanica, T. vogelii*, and *P. aduncum* were strongly synergistic at the LC95 level, except for the mixture of *T. vogelii*: *B. javanica*: *P. aduncum* (1: 0.5: 2.5) and a mixture of *T. vogelii* and *B. javanica* (5: 1) which is weakly synergistic.

### Table 3. Effect of mixed extract nanoemulsion of *Tephrosia vogelii* and *Piper aduncum* on the food utilization efficiency index of *Crocidolomia pavonana* larvae

| Concentration (%) | Growth consumption (mg/hari) | Growth rate (mg/days) | Efficiency of food utilization (%) |
|------------------|-------------------------------|----------------------|----------------------------------|
|                  | LK   | LKR  | LP    | LPR   | DC   | EMK | EMC  |
| Control          | 0,037a | 1,312a | 0,012a | 0,423a | 0,003a | 42,4a | 63,9a |
| 0,03/LC25        | 0,027b | 1,158a | 0,010a | 0,423a | 0,005a | 120,7a | 128,2a |
| 0,04/LC50        | 0,026b | 2,061a | 0,007b | 0,420a | 0,005a | 30,07a | 23,06a |

*Figures followed by the same letter in the same column are not significantly different according to the 5% LSD advanced test.

**Information:**

LK: Consumption Rate  
LKR: Relative Consumption Rate  
LP: Growth Rate  
LPR: Relative Growth Rate  
DC: Digestibility  
EMK: Efficiency of Food Consumed  
EMC: Efficiency of Digestible Food

### 4. Conclusions

Based on the research that has been done, it can be concluded that the nanoemulsion mixture of *T. vogelii* and *P. aduncum* extracts is synergistic, effective, and efficient to be used as an alternative in *C. pavonana* pest control. In order to obtain greater benefits, the mixed nanoemulsion should be tested for its effectiveness against other pests, its safety against natural enemies, and its effectiveness in controlling pests in the field.

### 5. Acknowledgement

Special thanks to Andalas University for the academic and technical support to complete this research. Also, for Funding through professor acceleration scheme (T/5/UN.16.17/PP.KP-KRP2GB/LPPM/2019).

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