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Surface Modification of Materials by Atmospheric-Pressure Plasma to Improve Impregnation with Essential Oils for the Control of *Tropilaelaps* Mites in Honeybees (*Apis mellifera*)

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Abstract: In this research, the absorption and release rate of the essential oil, *Amomum krervanh*, by seven different materials were evaluated. Cardboard showed the highest EO absorption capacity (0.93 ± 0.0052 µL of oil/mg of dry cardboard) followed by balsa wood and drawing board with the EO absorption of 0.77 ± 0.043 and 0.62 ± 0.010 µL of oil/mg of dry material, respectively. The results also demonstrated that cardboard had the highest EO retention (52.84 ± 0.687% after 20 min of analysis). Additionally, the essential oil was released from the drawing board and cardboard at the same rate during the observation period. Surface modification of drawing board and cardboard was performed using atmospheric-pressure plasma for enhancing the material properties for *Tropilaelaps* control. The absorption capacity of cardboard was decreased after plasma treatment at an argon flow rate of 0.25 and 0.5 Lpm for 60 s/cm^2. However, the atmospheric-pressure plasma did not change the EO release property of these materials. Scanning electron microscopy analyses indicated a fractured and scaly surface after plasma treatment of gas flow rate at 0.5 Lpm and 1.0 Lpm for 30 s/cm^2. The surface chemical composition of materials was not altered following plasma treatment. Although the number of mite-infested brood cells did not differ significantly between treatment groups at the end of the field experiment, *Tropilaelaps* spp. populations in the plasma-treated cardboard impregnated with EO 5% (v/v) treatment were lower on days 7–14 of the experiment. Thus, parameters related to the atmospheric-pressure plasma should be further optimized to improve the material surfaces for use with essential oils to control honeybee mites.

Keywords: atmospheric-pressure plasma; surface modification; essential oil; absorption capacity; release rate; *Tropilaelaps* mite control

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1. Introduction

Tropilaelaps spp. mites are a serious ectoparasitic mite of honeybees, *Apis mellifera*, in Thailand and Asia. They reproduce rapidly and have a shorter phoretic stage than another honeybee mite, *Varroa* spp. [1]. Many factors, including the hygienic behavior of honey bees, acaricide-use regime, the beekeeper's management, and climate are all considered to influence the prevalence of *Tropilaelaps* spp. [2–4]. The intensity of *Tropilaelaps* spp. parasitism is highest during honeybee brood rearing periods [4]. In Thailand, the prevalence of mites can vary among the regions. Mite populations are consistently present in the northern region of Thailand including Nan, Chiang Rai, and Phrae provinces [5]. The prevalence is usually high by the end of rainy and all winter seasons according to Thai

beekeepers. Conversely, a high population of *Tropilaelaps* spp. was observed in autumn (86%) and it was less in winter (15%) in China [6]. Currently, synthetic chemical acaricides are widely used to control *Tropilaelaps* spp. such as amitraz, fluvalinate and flumethrin [2,7–9]. Formic acid is also applied in the colony to control the honeybee mites. However, these acaricides are not always effective against *Tropilaelaps* spp. [9]. Moreover, the application of some acaricides in colonies can affect queen performance and colony growth and residual chemicals are also detected in beehives and bee products [10–13]. Currently, alternative strategies including the use of essential plant oils have shown promise in controlling both honeybee mites, *Varroa* spp. and *Tropilaelaps* spp. [14–16]. Among the tested alternative products, some essential oils demonstrated high acaricidal activity against *Tropilaelaps* spp. with less toxicity to the honey bees [16]. Although essential oils have shown efficacy against *Tropilaelaps* spp. mites under laboratory conditions, the test of essential oils under colony conditions has demonstrated inconsistent efficacy [16]. The media materials and materials' surface functionalization could affect the release rate and distribution of essential oils within the colony. Different materials, such as a cardboard strip, grease and a sponge were used for testing as potential application methods to deliver the essential oil of cardamon *(Amomum krervanh)*. The results showed that cardboard was a simple and suitable media to distribute the essential oil in honeybee colonies [16]. In order to improve the efficient distribution of essential oil, the surface of materials may need to be modified. Of the various methods for surface modification, non-thermal plasma (NTP) technology has gained attention as a new surface modification method.

NTP is an electrically energized matter in a gaseous state at atmospheric pressure. Plasma is considered the fourth state of matter and is composed of ionized particles such as electrons, ions, neutrals, excited species, and reactive species [17,18]. The surface properties of the materials can be modified through these ionized particles. NTP is widely used to modify the surface properties of polymers [19–21].

In our previous work, three materials were investigated for the distribution of essential oils [16]. Nevertheless, the control of *Tropilaelaps* spp. mites was still inefficient under the environmental conditions tested. In this study, the characteristics of various materials were investigated for essential oil distribution. The materials were surface modified with the non-thermal plasma to improve the essential oil adsorption and release rate of the treated cardboards for controlling parasitic mites.

2. Materials and Methods

2.1. Essential Oil Extraction

The dried fruit of *A. krervanh* was obtained from an herbal market in Chiang Mai, Thailand. The plant material was ground using a blender to produce a uniform powder. Approximately, two hundred grams of the powdered plant materials were added to 1.5 L of distilled water. The essential oil (EO) was thus extracted using a hydro-distillation method for 3 h or until no essential oil remained. The different batches of essential oil were collected into a single solution, and anhydrous sodium sulfate was added to remove excess water [14]. The extraction process was run several times until a sufficient quantity of oil was obtained for the experiments. The extracts were then stored at 4 °C until used.

2.2. Materials

Commercial samples of cardboard, balsa wood, drawing board, teak wood, plywood, brown plywood, and popsicle stick wood were purchased from a store in Phrae, Thailand. The pieces sized 2 cm × 5 cm of each material were cut for the laboratory experiment. The properties (essential oil absorption capacity, holding capacity and release rate) of all material samples were analyzed as described below. Then, two of seven materials were chosen for atmospheric-pressure plasma treatment based on their essential oil absorption capacity, holding capacity, and release rate.

2.3. Atmospheric-Pressure Plasma Treatments

Surface modification of the materials was carried out with an atmospheric-pressure plasma jet system based on gliding arc discharge in air, as shown in Figure 1. Materials were placed under an atmospheric-pressure plasma jet nozzle with a working distance of 1.0 cm. Surface modification of materials was performed by the generation of plasma discharge over the material surface as a function of argon (Ar) gases at a fixed voltage of 1.4 kV and the gas flow rate was monitored by a mass-flow controller and maintained at 0.25 and 0.5 liters per minute (Lpm) for 30 and 60 s/cm². Three pieces of each material were used as replicates.

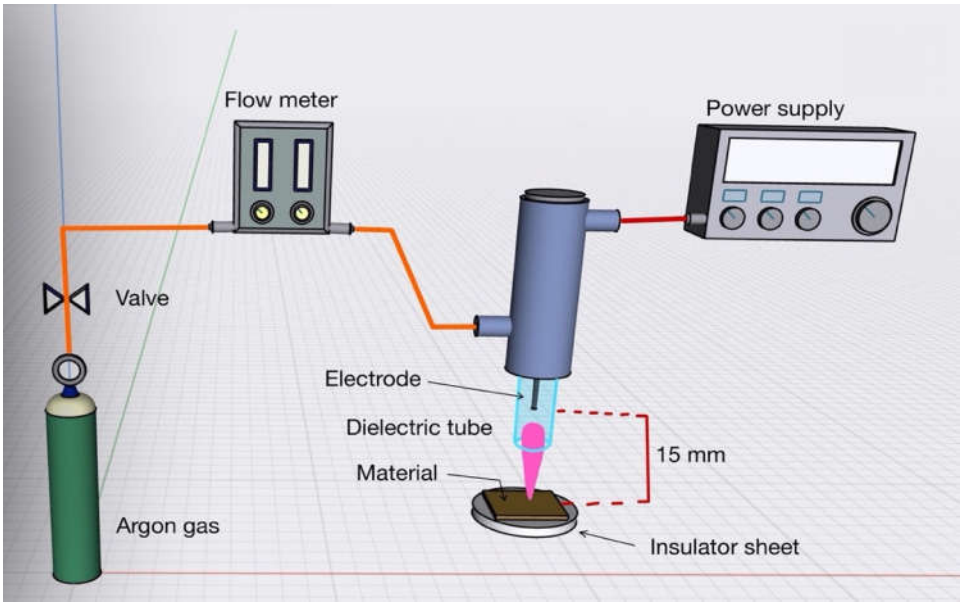


Figure 1. Schematic diagram of the atmospheric-pressure plasma jet system for the surface modification of materials.

2.4. Impregnation Process

After atmospheric-pressure plasma treatment, materials were immersed in 100 mL of essential oil of *A. krervanh* at room temperature in a tightly closed container to prevent evaporation and were kept in the dark for 24 h. Then, each material was removed from the essential oil and wiped with filter paper to remove any excess essential oil [22].

2.5. Analysis of Material Properties

2.5.1. Essential Oil Absorption Capacity

The dry and impregnated (soaked) materials were weighed using an analytical balance (accurate to 0.0001 g). The amount of adsorbed EO was calculated based on the standard curve. The results were presented as μL of EO/mg of dry material [22].

2.5.2. Essential Oil Holding Capacity

After materials were impregnated in EO, wiped with filter paper, and weighed in the impregnation process, all samples were then centrifuged at 2000 rpm for 5 min × 5 times. Every 5 min, the samples were weighed and the EO holding capacity values were calculated as EO holding capacity (%) = $((W_{wet} - W_{dwet})/W_{dry}) \times 100$, where W_{wet} is the weight of the EO impregnated material; W_{dwet} is the weight of the EO impregnated material after centrifugation and W_{dry} is the dry weight of the material before impregnation. The experiment was run in triplicates [22].

2.5.3. Essential Oil Release Rate

After the impregnation process, each material sample was incubated in 10 mL of liquid paraffin (solvent to dissolve EO) at room temperature for 72 h in sealed beakers. The solutions were mixed and 400 μL of the obtained solution was taken out for absorbance measurement using a spectrophotometer at 300 nm. Then, solutions were put back into the beaker at each time interval. The EO release rate was measured at 4, 8, 24, 32, 48, 56, and 72 h.

The experiment was conducted in technical triplicates. The absorbance values obtained were used to calculate the concentration of EO released (μL/mL). The calculation was performed based on the standard curve with known concentrations of EOs (absorbance vs. EO concentration) [22].

2.5.4. Surface Characterization

The changes in surface morphology of materials were evaluated by using a scanning electron microscope (JEOL, JSM-6010LV, Tokyo, Japan). The Fourier transform infrared spectroscopy (Perkin Elmer, Spectrum GX, Waltham, MA, USA) was used to examine the chemical structure of the material surface (The Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand).

2.6. Essential Oil Impregnated Materials for Tropilaelaps spp. Mites Control under Field Conditions

An optimal material sample treated with an atmospheric-pressure plasma was chosen to test in controlling *Tropilaelaps* spp. mites under field conditions. Each material was cut into the size of 2.5 × 10 cm and treated with atmospheric-pressure plasma by using Ar as the working gas at a flow rate of 0.50 Lpm for 30 s/cm^2. The distance between the plasma jet nozzle and material surface was 1.0 cm and a voltage was fixed at 1.4 kV (Figure 1). Each material sample was soaked in 5% (*v*/*v*) of essential oil dissolved in liquid paraffin for 24 h.

The colony-level field experiment was carried out in Chiang Mai, Thailand, from January to March 2021. Colonies used in these studies contained 2–3 frames of sealed brood, 1–2 frames of unsealed brood, and 1 frame of pollen and honey. Each hive consisted of a single Langstroth deep box with 10 frames with adult bees covering 7–9 frames. All hives had an active queen, and the most uniform hives were selected from all hives in the apiary. The honeybee colonies had not been treated for mite in the previous 4–6 months. Colonies were evaluated for adult bee and mite populations. Counting the adult bee population consisted of a visual inspection of every comb and frames covered in adult bees were estimated to the nearest 0.5 frame coverage (1 = full adult bee coverage). To determine mite infestation rate, a total of 100 cells per colony were opened. Groups of 10 cells in a line were uncapped and the observer worked at random across the sealed brood area, normally opening 50 cells on each two sides of a single brood frame [9]. Cardboard was chosen as the EO delivery system for the field study. Four treatments consisted of untreated cardboard impregnated with 5% (*v*/*v*) of EO, plasma-treated cardboard impregnated with 5% (*v*/*v*) of EO, untreated cardboard impregnated with 65% (*v*/*v*) of formic acid (positive control), and untreated cardboard impregnated with liquid paraffin (negative control). Mite levels were determined for each colony and then treatments were assigned using a stratified random design in which colonies were ranked, high to low mite infestation, and treatments assigned down the rank in groups of nine, to ensure balanced mite infestations across treatment groups. Two strips of each treatment were applied weekly for four weeks. Colonies were evaluated for adult bee population density and mite infestation using the methods described above, at four-time intervals following the initiation of treatments [9].

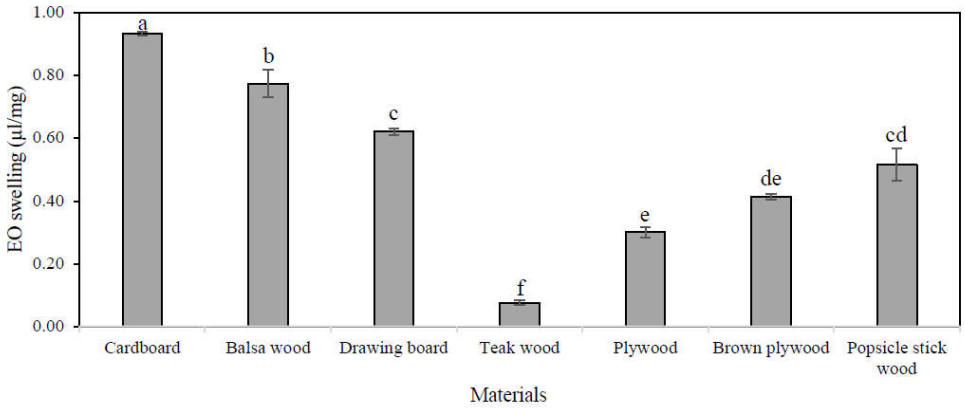
2.7. Statistical Analysis

The data were statistically analyzed using JMP version 11.2 for Mac (SAS Institute Inc., Cary, NC, USA). The normality of the data was checked using the Shapiro–Wilk test. One-way ANOVA followed by Tukey-HSD analysis was applied when data were normally distributed. The non-parametric Kruskal–Wallis test was used to determine if all data had significant differences followed by a Steel–Dwass posthoc multiple comparisons test to separate means when significance was found. The results of statistical analyses were considered significant if they produced p-values <0.05. The holding capacity and release rate of essential oil were compared between materials at each time interval. The number of mite-infested brood cells and the adult bee population in honey bee colonies were compared between treatments at each time observation period.

3. Results

3.1. Essential Oil Absorption Capacity, Holding Capacity, and Release Rate of Materials

Seven different materials (cardboard, balsa wood, drawing board, teak wood, plywood, brown plywood, and popsicle stick wood) were tested for essential oil absorption capacity, holding capacity, and release rate. The quantity of essential oil absorbed by the materials was significantly different (Kruskal–Wallis test, $p < 0.0001$) (Figure 2a). Cardboard displayed the highest essential oil absorbency (0.93 ± 0.0052 µL of oil/mg of dry cardboard) (Figure 2a). Balsa wood and drawing board were able to absorb 0.77 ± 0.043 and 0.62 ± 0.010 µL of oil/mg of dry material, respectively. The lowest absorption capacity was found in teak wood with the value of 0.08 ± 0.0073 µL of oil/mg of dry teak wood. The order of essential oil absorption capacity of different materials was ranked as cardboard > balsa wood > drawing board > popsicle stick wood > brown plywood > plywood > teak wood.



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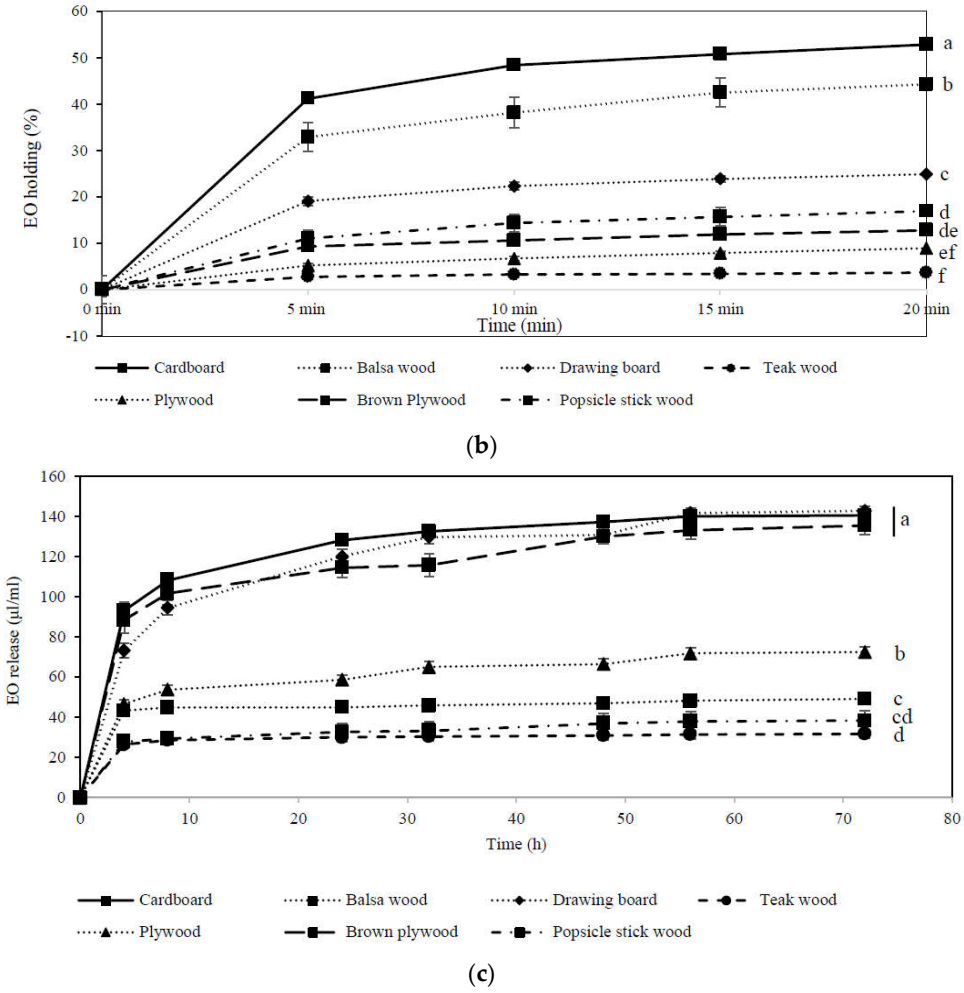


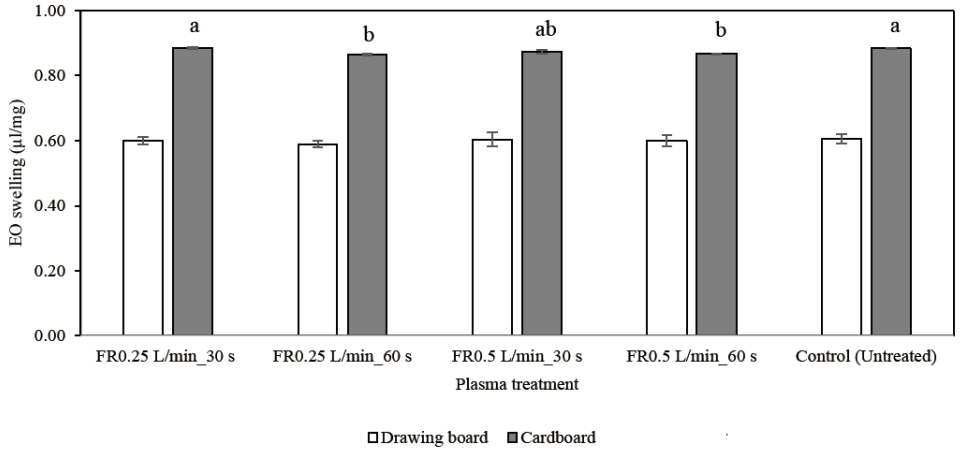
Figure 2. Absorption capacity (**a**), holding capacity (**b**), and release rate (**c**) of *A. krervanh* EO by different materials. Data shown are the means ± standard deviation. Different lowercase letters represent significant difference ($p < 0.05$). Comparisons of holding capacity and release rate of essential oil were made between materials at each time interval.

The essential oil holding capacity of materials is shown in Figure 2b. The essential oil holding capacity of cardboard was significantly higher than the other materials at each time interval in the range of 41.22 ± 0.787 − 52.84 ± 0.687% (Kruskal–Wallis test, $p < 0.0001$). For teak wood, essential oil holding capacity was the lowest with 2.79 ± 0.26 − 3.67 ± 0.33% in 20 min of analysis. The results demonstrated that the essential oil molecules were gradually released from each material (Figure 2c). Cardboard, brown plywood, and drawing board showed the higher release rates of essential oil (ANOVA, $p < 0.0001$). The essential oil was released at 93.17 − 139.94 µL/mL, 88.53 − 135.41 µL/mL and 73.16 − 142.74 µL/mL in cardboard, brown plywood and drawing board, respectively, after 72 h of incubation.

3.2. Effect of Atmospheric-Pressure Plasma on the Property of Materials

Cardboard and drawing board were chosen to be treated with atmospheric-pressure plasma based on their essential oil absorption capacity, holding capacity, and release rate obtained in the previous experiment. The effect of atmospheric-pressure plasma on essential oil absorption capacity and release rate at an argon flow rate of 0.25 and 0.5 Lpm for 30 and 60 s/cm² is shown in Figure 3. For the cardboard, EO absorption capacity significantly decreased after plasma treatment at an argon flow rate of 0.25 and 0.5 Lpm for 60

s/cm² (ANOVA, $p = 0.0048$) (Figure 3a). The values were about 0.8643 ± 0.003 and 0.8673 ± 0.002 µL of oil/mg of dry material for plasma-treated cardboard at an argon flow rate of 0.25 and 0.5 Lpm for 60 s/cm², respectively. However, plasma treatment did not affect the EO absorption capacity of drawing board (ANOVA, $p > 0.05$).



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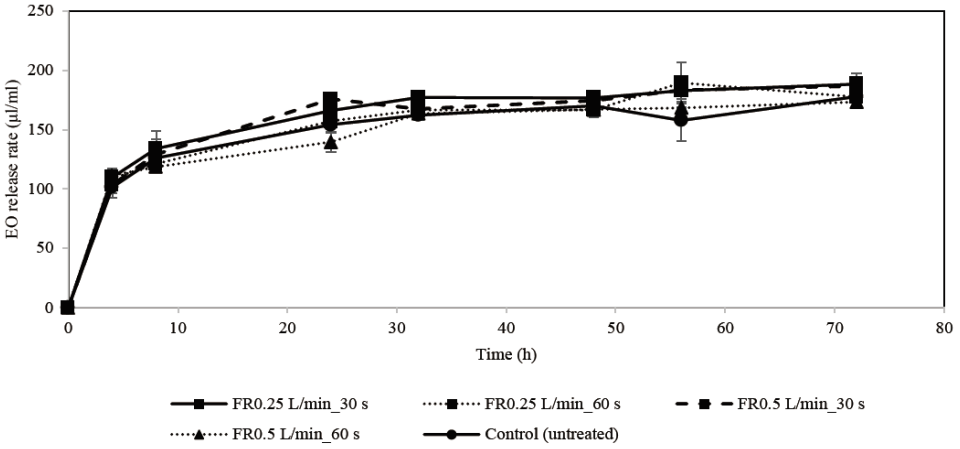

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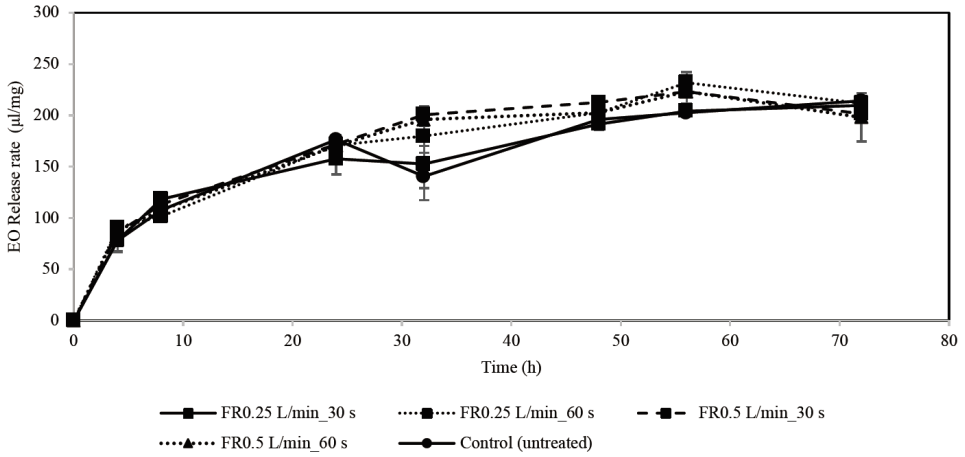


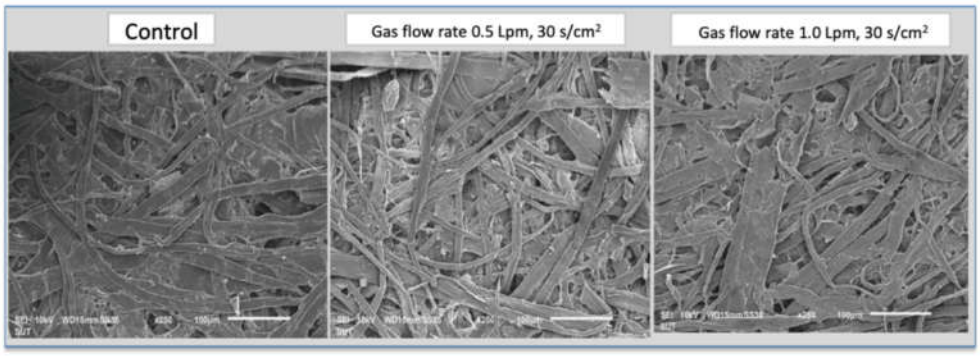
(**c**)

Figure 3. Absorption capacity of *A. krervanh* EO by cardboard, and drawing board (**a**), and release rate of EO by cardboard (**b**) and drawing board (**c**) after plasma treatment. Data shown are the means ± standard deviation. Different lowercase letters represent significant difference ($p < 0.05$). The EO absorption of each material was compared between plasma treatments. Comparisons of the release rate of essential oil were made between plasma treatments at each time interval.

The essential oil release rate of materials was also analyzed. It showed that essential oil molecules were dramatically released from both materials after 4 h of incubation and were slow released until 72 h (Figure 3b,c). EO release rate was not significantly different after the plasma treatment of both cardboard and drawing board at all time intervals, except at 24 h, plasma treatment at gas flow rate at 0.5 Lpm for 60 s/cm^2 decreased the EO release rate of cardboard (ANOVA, $p = 0.0053$) (Figure 3b). The essential oil molecules were released from cardboard and drawing board at about 180.88 µL/mL and 206.92 µL/mL, respectively, after 72 h of incubation.

3.3. Surface Modification of Material after Atmospheric-Pressure Plasma Treatment

Surface morphology of cardboard and drawing board before and after plasma treatment is shown in Figure 4. As can be seen from the SEM micrographs, a smooth surface of untreated cardboard and drawing board is observed (Figure 4a). An atmospheric-pressure plasma treatment had no significant influence on the material surface morphology. However, both materials showed a fractured and scaly surface after plasma treatment of gas flow rate at 0.5 Lpm and 1.0 Lpm for 30 s/cm^2. The surface modification was clearly observed when the higher gas flow rate was applied (1.0 Lpm).



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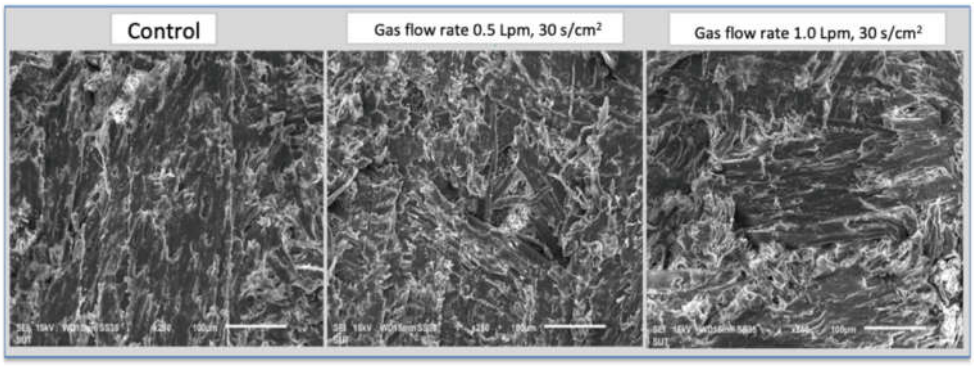
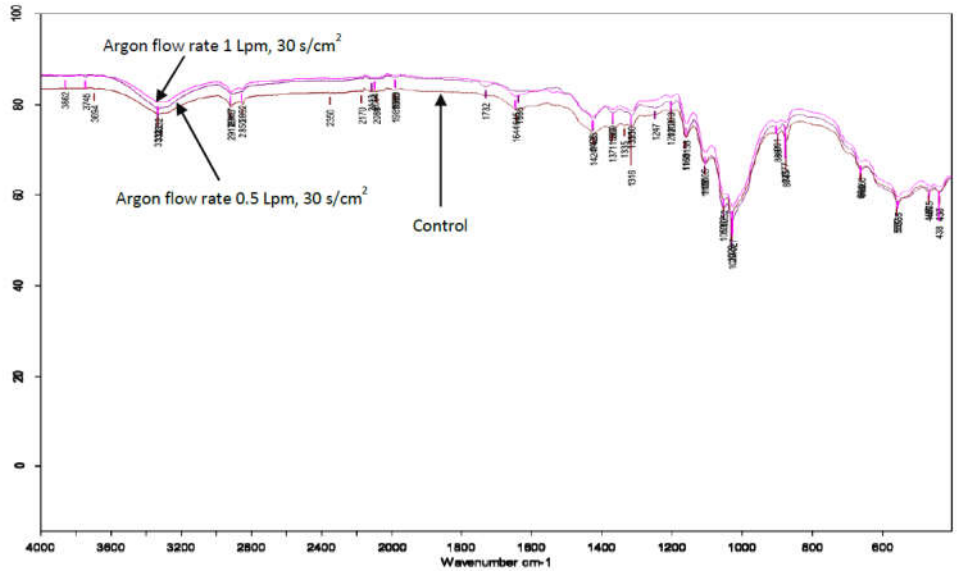


(**b**)

Figure 4. Scanning electron microscope (SEM) images of cardboard (**a**) and drawing board (**b**) after plasma treatment (magnification ×250).

The surface chemical composition of the untreated and plasma treated cardboard and drawing board was analyzed by Fourier transform infrared (FTIR). The results displayed that the plasma treatment of Ar flow rate at 0.5 Lpm and 1.0 Lpm for 30 s/cm² did not change the chemical composition of the materials surface (Figure 5).



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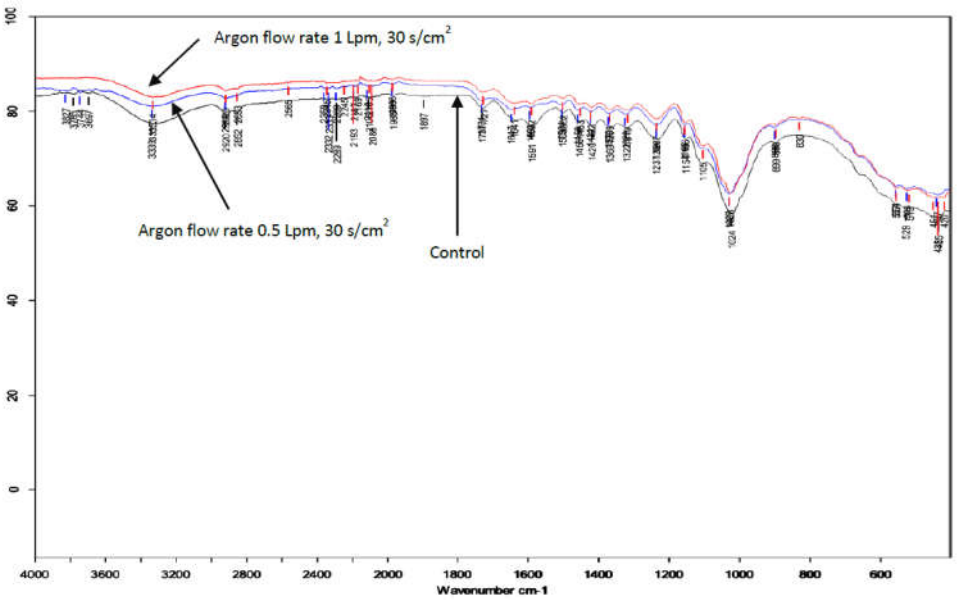
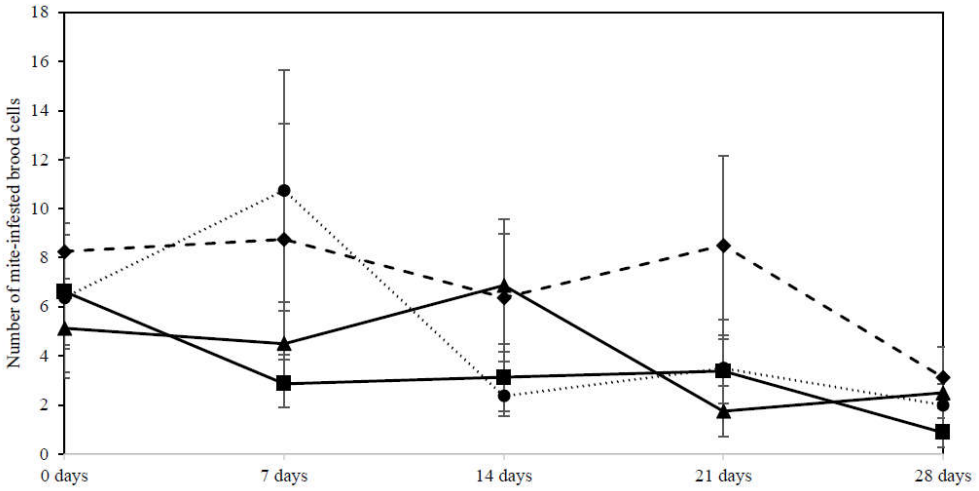


(**b**)

Figure 5. FTIR spectra of cardboard (**a**) and drawing board (**b**) after plasma treatment.

3.4. Efficacy of Plasma Treated Cardboard Impregnated with Essential Oil on Tropilaelaps spp. Mite Control under Field Conditions

Cardboard pieces after the plasma treatment of gas flow rate at 0.50 Lpm for 30 s/cm^2 impregnated with 5% (*v*/*v*) of essential oil were tested for an acaricidal activity under field conditions in colonies with 5–7 frames of adult bees. The results showed that essential oil of *A. krervanh* decreased the mite populations throughout the experiment (Figure 6a). Plasma treatment had no significant impact on cardboard properties that could help to release the essential oil for *Tropilaelaps* spp. control (Kruskal–Wallis test, $p > 0.05$). Although the mite population was increased on day 7, it was dramatically dropped on day 14 and the mite infestation remained low until the end of the experiment when colonies were treated with plasma-treated cardboard impregnated with essential oil. The number of mite-infested brood cells on day 28 in the colony receiving untreated and plasma-treated cardboard impregnated with essential oil was 0.875 ± 0.611 and 2.000 ± 0.845, respectively. Mite infestation in colonies treated with 65% (v/v) of formic acid did not really change after day 21 of the experiment; however, the number of mites was reduced (3.125 ± 1.260) on day 28. The adult bees of all treatment groups were about 6–8 frames throughout the experiment and there were no significant differences between treatments at each time interval (Figure 6b).



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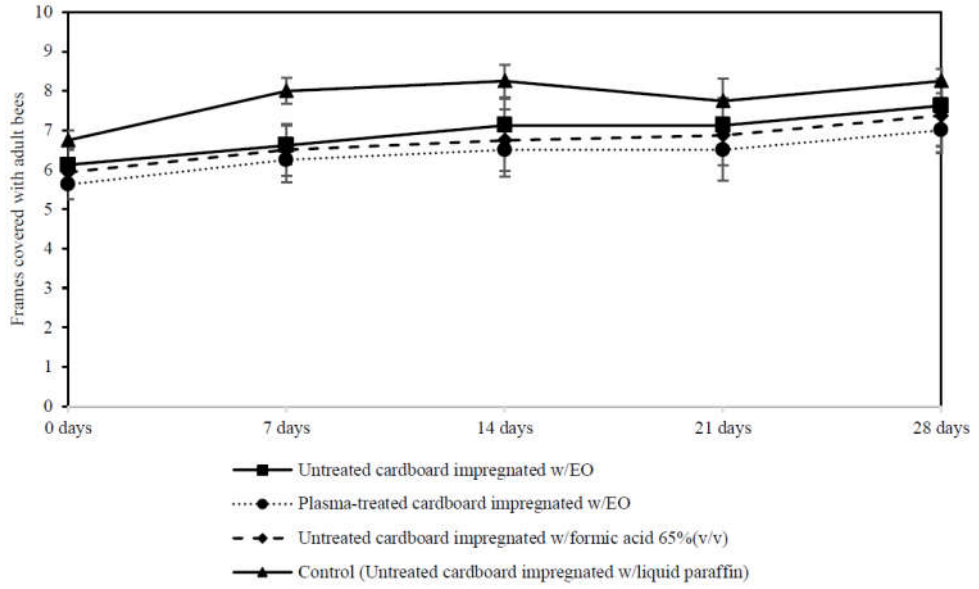

(**b**)

Figure 6. Number of mite-infested brood cells (**a**) and average number of frames covered with adult bees (**b**) in honey bee colonies (n = 9/treatment group) after *A. krervanh* EO treatment using plasma-treated cardboard (5% (w/v)) compared to controls (untreated cardboard impregnated with EO and untreated cardboard impregnated with liquid paraffin) and untreated cardboard impregnated with formic acid (65% (v/v)) at each observation period (before treatment and at 7, 14, 21 and 28 days after treatment) (Kruskal-Wallis H and Steel-Dwass test, $p > 0.05$).

4. Discussion

Several essential oils and natural plant extracts have been reported to have acaricidal activity against honeybee mites [14–16]. Although a high potential for mite control has been observed under laboratory experiments, essential oils were less effective when tested in honeybee colonies under field conditions [16]. Effective concentrations and application methods of essential oil, along with varying environmental conditions, could be important factors affecting efficacy [16]. Increasing mite control efficiency could be improved with different absorption and release rates of essential oil from media materials [23,24]. In this study, media type selection and surface modification of the media by atmospheric-

pressure plasma for essential oil absorption and release improvement were investigated. Cardboard and drawing board showed improved properties with cardamon essential oil absorption and release rate compared to the other wood medium. This could be due to the specific characteristic of these media such as the porous nature of the material or other aspects of their composition. Other media materials used in this study were hardwood, in which the essential oil had difficulty being absorbed. However, grease and a sponge have been used as material to distribute the essential oil of *A. krervanh*. The properties of both materials did not increase the release rate and distribution of essential oil [16]. Therefore, only cardboard and drawing board materials were chosen for further study, with surface modification using plasma technology. The performance and functionality of the materials depends on their physical and chemical properties. Especially, surface properties are crucial to their performance and functionality. Plasma, the complex mixture of ions, radicals, free electrons, and highly energetic molecules is a novel method for developing various materials. Since the contact between plasma and material surfaces can cause important changes to their surface properties [25]. Our results showed that the cardboard and drawing board's properties on essential absorption and release rates did not significantly change after using plasma treatment, demonstrating the incapability of plasma treatment under the condition tested in this study for modifying the surface property of cardboard and drawing board. The plasma duration and gas flow rate applied in this study may not be sufficient for surface energy effect changes in some of the media tested. However, the surface of plasma treated cardboard and drawing board was changed. Surface modification by atmospheric-pressure plasma that occurred in this study would not be the effect of thermal damage because non-thermal plasma was used. The changes might be due to the etching effect of plasma treatment. Plasma etching could alter the surface energy by the incorporation or formation of chemical groups during the interaction of plasma particles with the material surface [26]. The main elements in the plasma that are responsible for etching effect are positive ions and protons, with the ability to break primary chemical bonds and induce cross-linking [27]. Therefore, the experiment parameters of plasma, such as power density, voltage, frequency, gas type and flow rate, working distance of plasma jet nozzle and duration, should be further investigated for a cost-efficient surface modification. Recently, non-thermal plasma has been widely used to modify the surface of various materials for improving and functionalizing the surface properties. Non-thermal plasma (NTP) has been used to develop the surface properties of hydrogels consisting of carboxymethyl guar gum and chitosan such as surface wettability, pH-responsive swelling, nanotopography and antibacterial activity, which could be effective for various biomedicine applications [28]. Moreover, plasma technology is extensively used for polymer surface modification and synthesis [17,25,29,30]. Plasma surface modification using air/He mixture as gas type has been found to be an effective method for enhancing the pigment adhesion and color strength of polyester fabrics [19]. López-García et al. (2018) showed that non-thermal plasma was suitable for the surface modification of polytetrafluoroethylene (PTFE) [20]. Especially, air plasma treatment changed the superhydrophobic character of PTFE. Moreover, plasma technology used for nanotexturing and the surface functionalization of polymers have been reviewed [21,31–33].

Although non-thermal plasma sources used in this work did change the surface morphology of cardboard and drawing board, these changes were not sufficient to enhance the surface properties of materials for *Tropilaelaps* spp. mite control. Therefore, additional parameters related to plasma treatment should be further investigated. Material types and suitable solvents for essential oil might also be important factors for essential oil distribution in a colony. Lastly, non-thermal plasma could be a useful technology to improve both honeybee parasitic mites and disease control in beekeeping.

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preparation, V.C., T.B., J.S.P., T.D. and C.S.; writing—review and editing, V.C., T.B., J.S.P., T.D. and C.S.; visualization, V.C., T.B.; supervision, V.C., J.S.P.; project administration, V.C., and C.S.; funding acquisition, V.C., T.D. and C.S. All authors have read and agreed to the published version of the manuscript.

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