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Biological transformed cannabinoids bee pollen: A symbiosis approach on *Apis mellifera* raising protocol in *Cannabis sativa* L. (Hemp) cultivar in Samoeng District, Chiang Mai, Thailand

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Abstract

Honey bees (*Apis mellifera*) and Hemp plants (*Cannabis sativa* L.) have been recognized as boosters in the world economy. Hemp pollens collected by honey bees for their nutrients and cannabinoids discrete from cannabis plants have not been synergized in business management. A symbiosis approach was adopted in this novel experimental method. This research aimed to investigate the presences of Cannabinoids (CBD, THC and CBN) via a prototype of bee-raising protocol with the different artificial fed supplements, foraging on the hemp plantation to collect hemp pollens. Theoretically, in-hived stored hemp pollen shall embrace active compounds as being abundance in hemp plants, thru biological transformation process. In the field experiment, seed production hemp cultivated in Samoeng District, Chiang Mai was fully covered by mosquito net to confine honey bees during male flowering. The extraction of those cannabinoids of in-hived stored pollen samples and their contents were conducted by GC-MS technique. A repeated measure ANOVA model was conducted for statistical analysis. The experimental research results indicated the first ever found of cannabinoids presented in in-hived stored pollen samples of all treatments. The statistical analysis among all treatments contained different means of detected Cannabinoids (CBD, THC and CBN) contents, which were statistically significant (P-value < 0.05) where the significant level of 95%. The results were remarkable as shown due to presence of CBD, THC and CBN, as bioactive compounds, in in-hived honey bee pollens that would enlighten an opportunity in managing bee as hemp plantation either for further academic or business purposes.

Keywords: *Cannabis sativa* L. (Hemp), cannabinoids, *Apis mellifera* (Honey bee)
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1. Introduction

The phytochemical constituents such as high potency cannabinoids have been drawing the world attentions. Many investigations of pharmaceutical and medicinal properties of *Cannabis sativa* L. is drawing attentions from scientist worldwide [1]. Cannabinoids, i.e. Δ9-tetrahydrocannabinol (THC), Cannabidiol (CBD) and Cannabinol (CBN) have been studying for future uses. Since the discovery of cannabinoid receptors and the Endo-cannabinoid System (ECS) in human's immune cells in the mid of 20th century [2 – 5] enhance wider and deeper researches in many countries including Thailand. Recent studies have documented the importance of hemp pollen in supporting a diverse community of honey bees during periods of floral resource scarcity [6, 7]. A mass flowering hemp crops can support pollinator populations foraging [8, 9]. The presence of cannabinoids, particularly psychoactive Δ9-tetrahydrocannabinol (THC) and Cannabinol (CBN) in hemp pollen however does not likely to have an impact on bee development due to the lack of cannabinoid receptors in insects [10]. The research aimed to investigate Cannabinoids in *Cannabis sativa* L. (hemp) plant being transferred by means of honey bee raising protocol with the different artificial fed supplements into in-hive stored pollen using a symbiosis approach cum a biological transformation technique. The raising bees need to have sufficient nutrient foods combining of proteins collected from plant pollens and carbohydrates from plant nectars. Since hemp plants are naturally lack of sufficient

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nectar to maintain bee colony healthy, artificial supplements in forms of diluted honey and diluted sugar syrup were adopted in the separated treatments.

2. Material and method

2.1 Field experiment

A total of 9 hives of raising honey bees were moved to 5 Rai hemp plot where hemps were planted for seed and fibre production in Baan Khong Khark Luang, Samoeng, Chiang Mai, Thailand (Latitude: 18°53'45.75" North and Longitude: 98°42'23.31" East). Bees were raised in beehives with monitoring system. Each group of 3 beehives were distinguished into 3 treatments (Ts) as T1- kept in netted hemp plot and in-hive drop-fed with 1:1 diluted honey; T2- kept in netted hemp plot and in-hive drop-fed with 1:1 diluted sugar syrup; and T3- kept outside netted hemp plot without any artificial feeding as a control that let bees free forage in opened hemp field next to the netted hemp plot. The differences of bee's nectar supplement consumed, can be influenced to the quality of bee product yields.

2.2 Sample collection process

The in-hive-stored Pollens, during the male flowering peak, were collected from brood frames into prepared and sterilized vials on every other days (on September 14, 16 and 18 between 13.00 and 16.00 hours in volumes of approximately 10 grams from each hive for sufficient laboratory investigation.

2.3 Laboratory materials and methods

Using Gas Chromatography-Mass Spectrometry Method (GC-MS) [11 – 13] was conducted by the certified Central Laboratory (Thailand) Co., Ltd. (Chiang Mai Branch): CLT. **Instruments: Gas Chromatograph/Mass Spectrometry Detector (GC/MSD) for cannabinoids: Gas chromatography**: Agilent technologies made in China; Model 6890 N, Oven 100 °C hold 1 min, 10 °C/min to 300 °C hold 9.0 min., Post time 5 min. at 330 °C, Total run time 30 min., Helium carrier gas flow 1.0 mL/min, Column DB 5MS Agilent technologies made in USA. 0.25 mm × 30m × 0.25 micron of film thickness., Inlet split 20:1 volume of injection 1 uL, Inlet temperature 280 °C, Auxiliary temperature 280 °C; **Mass spectrometer detector**: Agilent technologies made in USA., Model 5973 inert, Scan mode 40 – 500 m/z, MS Quadrupole temperature 150 °C, MS Source temperature 230 °C; **Database Agilent technologies USA**: Wiley version 9; **Basic instrument**: Ultrasonic bath: BRANSON 3510 USA., Vortex mixer: Genie 2 USA., Water bath: Memmert WNE21 Germany, Freezer -20 °C: Sanyo Japan; **Reagent**: Hexane: (HPLC Grade) Labsan Ireland.; **Cannabinoids standard THC/CBD/CBN**: RESTEX (34014) USA. Sample accurate weight of 0.05xx – 0.10xx grams.

2.4 Statistical method

According to the field experimental design and the laboratory method as described in prior sessions, a set of data was designed in 3 different treatments (3 bee hive replicates in each treatment) and in-hive stored bee pollen samples were repeatedly measured or collected in 3 time-points [14]. The harvesting time-point crucially reflects on a maturity of quality bee product yields [15]. Therefore, the reliability of a data set was tested by using replicated bee hive Standard Deviations (SDs) dispersed relative to replicated bee hive Means. Finally, the repeated measures ANOVA technique was separately analysed on each set of detected CBD, THC and CBN data [16].

3. Results

3.1 Detections of cannabinoids (CBD, THC and CBN) in in-hive stored bee pollen samples

Using the repeated measures ANOVA model. The laboratory results shown the detected CBD, THC and CBN comparison by each measuring time-point of the sample collections shown in Table 1, 4 and 7, respectively. Means and Standard Deviations (SDs) of each treatment's replication as shown in Table 2, 5 and 8, respectively, were statistically analysed below.

Figure 1: Experimental *Cannabis sativa* L. cultivar and foraging honey bee.

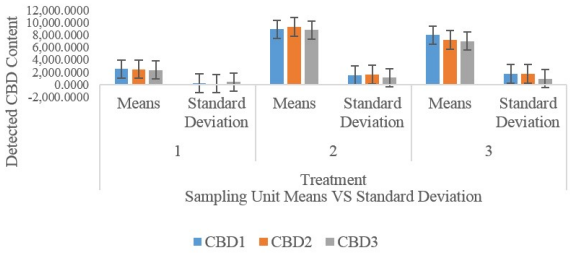

Figure 2: Observation on the measured values of detected CBD content (CBD1-3 measuring time-points) related to the sampling unit (each of 3 replicated beehives) means.

3.2 Detected cannabidiol (CBD) content (mg/kg) by treatment (experimental unit) classification.

As seen in above Table 1, 2 and Fig. 2, the datasets of detected CBD content were observed with the Stan-

Table 1. Detected cannabidiol (CBD) content (mg/kg) classified by treatment (experimental unit).

Treatment	Replication	Measure 1	Measure 2	Measure 3
1	1	2,348	2,335	1,954
(1:1 diluted honey fed)	2	2,703	2,600	2,428
	3	2,426	2,445	2,728
2	1	9418	10,903	10,152
(1:1 diluted sugar syrup fed)	2	10,209	9,470	8,021
	3	7,252	7,663	8,482
3	1	6,060	6,824	7,288
(Controlled)	2	8,786	5,768	6,005
	3	9,180	9,166	7,777

Table 2. Standard Deviation on the measured data of detected CBD content dispersed to the sampling unit (each of 3 replicated beehives) mean.

Measure #	Treatment					
	1 (Diluted honey fed)		2 (Diluted sugar syrup fed)		3 (Controlled)	
	Means	Standard Deviation	Means	Standard Deviation	Means	Standard Deviation
CBD1	2,492.3333	186.5646	8,959.6667	1530.8541	8,008.6667	1699.0542
CBD2	2,460.0000	133.1353	9,345.3333	1623.5936	7,252.6667	1739.0852
CBD3	2,370.0000	390.2461	8,885.0000	1121.2034	7,023.3333	915.1679

Table 3. Analysis of variance of detected cannabidiol (CBD) content.

Sources of Variation	Sum of Squares	Degrees of Freedom	Root Means Square (RMF)	$F_{(1,2\&3)}$	P-value
Experimental Unit (Treatment)	214,219,297.556	2	107,109,648.778	39.906	<0.0009
Discrepancy	16,104,120.444	6	2,684,020.074		
Sum of between Groups	230,323,418.00	8			
Time of Measure	722,644.222	2	361,322.111	0.422	0.665
Time of Measure * Exp. Unit	1,262,698.889	4	315,674.722	0.369	0.826
Discrepancy	10,276,331.556	12	856,360.963		
Sum of Within Group	12,261,674.67	18			
Sum Total	**242,585,092.67**	**26**			

dard Deviation of each replicated bee-hive in all treatments are dispersed closely to their means, in the same dispersion pattern. Therefore, they were statistically reliable for further statistical analysis of ANOVA.

3.3 Analysis of variance of detected cannabidiol (CBD) content

From the test, it was found that

Where F_1 = 39.906 It could be concluded that each experimental unit contains different means of detected CBD, P-value < 0.0009. When a pair test was given, the findings were that none of means differences of experimental unit 2 and 3 was found with greater means than experimental unit 1.

Where F_2 = 0.422 It could be concluded that none of different means was found in each measure of detected CBD, P-value = 0.665.

Where F_3 = 0.369 It could be concluded that none of different interaction within measure was found with experimental unit, P-value = 0.826.

Where the significant level in this experiment = 95%

The findings could be interpreted that among 3 different treatments, as the above statistical results showed; among the group of treatments, each treatment had different means of detected CBD which is statistically significant difference (P-value < 0.05). While none of the different means within each group revealed no statistically significant difference (by each measure and within each treatment P-value > 0.05). Therefore, measured data within a group of treatments were the same means of detected CBD. When comparing average means among all treatments (illustrated in Fig. 3 below); detected CBD content of treatment 2 (with diluted syrup fed and confined bees) and controlled treatment 3 (without feeding, with free foraging) were greater than treatment 1 (with diluted honey fed and confined bees). It was noticeable that under the same protocol of bee raising in order to manage a honey bee colony most ready for foraging *Cannabis sativa* L. (hemp); the artificial feeding with carbohydrate source (nectar) was influential in CBD content contained in bee produce yields.

3.4 Detected Δ9-tetrahydrocannabinol (THC) content (mg/kg) by treatment (experimental unit)

According to the observation on the measured values of detected THC content related to the means of each replicated hives of each treatment (Table 4, 5 and Fig. 4), the dataset of detected THC content was dispersed closely relative to their means in the same dispersion pattern. Therefore, these measured data set

Table 4. Detected Δ9-tetrahydrocannabinol (THC) content (mg/kg) classified by treatment (experimental unit).

Treatment	Replication	Measure 1	Measure 2	Measure 3
1	1	8.69	16.92	14.56
(1:1 diluted honey fed)	2	17.04	12.19	16.17
	3	17.46	14.41	17.05
2	1	8.62	7.75	2.75
(1:1 diluted sugar syrup fed)	2	3.32	1.05	1.00
	3	1.72	6.95	7.53
3	1	1.38	1.08	1.00
(Controlled)	2	4.50	3.51	1.00
	3	1.85	1.00	1.00

Table 5. Standard deviation on the measured data of detected THC content dispersed to the sampling unit (each of 3 replicated beehives) mean.

Measure #	Treatment					
	1 (Diluted honey fed)		2 (Diluted sugar syrup fed)		3 (Controlled)	
	Means	Standard Deviation	Means	Standard Deviation	Means	Standard Deviation
THC1	14.3967	4.9466	4.5533	3.6116	2.5767	1.6822
THC2	14.5067	2.3665	5.2500	3.6592	1.8633	1.4266
THC3	15.9267	1.2627	3.7600	3.3801	1.0000	0.0000

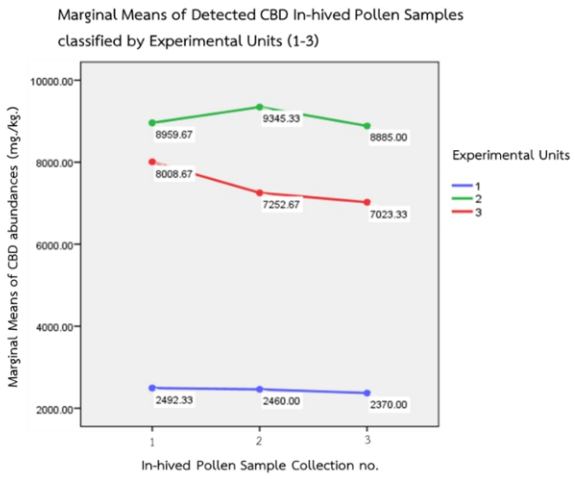

Figure 3: Means of detected Cannabidiol (CBD) illustrated by treatment (experimental unit).

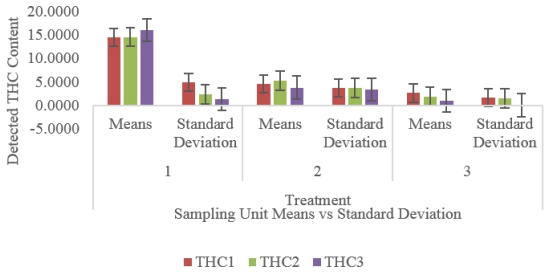

Figure 4: Observation on the measured values of detected THC content (THC1-3 measuring time-points) related to the sampling unit (each of 3 replicated beehives) means.

of detected THC contents are statistically reliable for the next statistical analysis of the Repeated Measures ANOVA test.

3.5 Analysis of variance of detected Δ9-tetrahydrocannabinol (THC) content

From the test, it was found that

Where $F_4 = 47.787$ It could be concluded that each experimental unit contains different means of detected THC, P-value ¡ 0.0009. When a pair test was given, the findings were that none of the means differences of experimental unit 2 and 3 was found with lesser means than experimental unit 1.

Where $F_5 = 0.034$ It could be concluded that no different means were found in each measure of detected THC, P-value = 0.967.

Where $F_6 = 0.347$ It could be concluded that none of different interaction within measures was found with experimental units, P-value = 0.841.

Where the significant level in this experiment = 95%

It could be interpreted that among 3 different treatments, as the above statistical results showed; among the group of treatments, each treatment had different means of detected THC which is statistically significant difference (P-value < 0.05). While none of the different means within each group showed no statistically significant difference (by each measure and within each treatment, P-value > 0.05). Therefore, measured data within a group of treatments had the same means of detected THC. When comparing average means among all treatments (Fig. 5); detected THC contents of treatment 2 (with diluted syrup in-hive fed and confined bees) and controlled treatment 3 (without feeding, with free foraging bees) were less than treatment 1 (with diluted honey in-hive fed and confined bees). Detected THC contents in nominal terms (mg/kg) were much less than those of CBD contents, comparatively caused by hemp plants are naturally less THC content.

Table 6. Analysis of variance of detected Δ9-tetrahydrocannabinol (THC) content.

Sources of Variation	Sum of Squares	Degrees of Freedom	Root Means Square (RMF)	$F_{(4,5\&6)}$	P-value
Experimental Unit (Treatment)	865.055	2	432.528	47.787	<0.0009
Discrepancy	54.307	6	9.051		
Sum of between Groups	919.36	8			
Time of Measure	0.528	2	0.264	0.034	0.967
Time of Measure * Exp. Unit	10.916	4	2.729	0.347	0.841
Discrepancy	94.467	12	7.872		
Sum of within group	105.91	18			
Sum Total	**1,025.27**	**26**			

Table 7. Detected cannabinol (CBN) content (mg/kg) classified by treatment (experimental unit).

Treatment	Replication	Measure 1	Measure 2	Measure 3
1	1	1.00	1.35	1.00
(1:1 diluted honey fed)	2	1.02	0.00	1.00
	3	0.00	1.00	1.00
2	1	1.60	2.00	2.26
(1:1 diluted sugar syrup fed)	2	1.34	1.80	2.18
	3	1.30	1.03	1.00
3	1	1.13	1.68	1.69
(Controlled)	2	1.84	1.68	1.23
	3	1.69	1.69	1.00

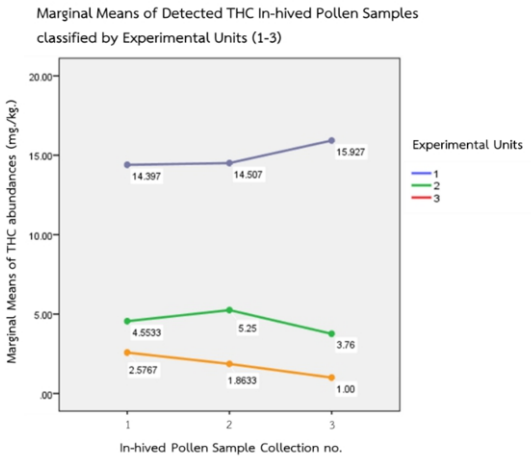

Figure 5: Means of detected Δ9-tetrahydrocannabinol (THC) illustrated by treatment (experimental unit).

3.6 Detected cannabinol (CBN) content (mg/kg) by treatment (experimental unit) classification

The above Table 7, 8, together with Fig. 6 showed that the dataset of detected CBN content was dispersed closely relative to their means, as in the same dispersion pattern. As a result, statistically reliable data set was confident for further statistical analysis of the repeated measures ANOVA test.

3.7 Analysis of Variance of detected cannabinol (CBN) content

From the test, it was found that

Where $F_7 = 6.290$ It could be concluded that each experimental unit contains different means of detected CBN, P-value = 0.034. When a pair test was given, the findings were that the Experimental units 2 and 3: means did not differ. Experimental units 1 and 3: means did not differ. Experimental unit 2: means was found with greater than those of experimental unit 1

Where $F_8 = 0.392$ It could be concluded that no different means were found in each measure of CBN, P-value = 0.684.

Where $F_9 = 0.674$ It could be concluded that no different interaction within measures was found with experimental units, P-value = 0.623.

Where the significant level in this experiment = 95%

It could be interpreted that among 3 different treatments, as the above statistical results showed; among the group of treatments, each treatment had different means of detected CBN which is statistically significant (P-value < 0.05). While none of different means within each group showed no statistically significant difference (by each measure and within each treatment, P-value > 0.05). Therefore, measured data within a group of treatments were the same means of detected CBN. When comparing average means among all treatments; detected CBN contents of treatment 2 (with diluted syrup in-hive fed and confined bees) was greater than treatment 1 (with diluted honey in-hive fed and confined bees) (Fig. 7). While average means of detected CBN between treatment 1 and treatment 2 as well as those between treatment 1 and controlled treatment 3 were not different. While detected CBN content in nominal terms (mg/kg) were much lesser than those of detected CBD content, and as in small amount as of detected THC content (Fig. 3, 5 and 7).

Table 8. Standard deviation on the measured data of detected CBN content dispersed to the sampling unit (each of 3 replicated beehives) mean.

Measure #	Treatment					
	1 (Diluted honey fed)		2 (Diluted sugar syrup fed)		3 (Controlled)	
	Means	Standard Deviation	Means	Standard Deviation	Means	Standard Deviation
CBN1	.6733	.5832	1.4133	.1629	1.5533	.3742
CBN2	.7833	.7006	1.6100	.5122	1.6833	.0058
CBN3	1.0000	0.0000	1.8133	.7055	1.3067	.3513

Table 9. Analysis of variance of detected Cannabinol (CBN) content.

Sources of Variation	Sum of Squares	Degrees of Freedom	Root Means Square (RMF)	$F_{(7,8\&9)}$	P-value
Experimental Unit (Treatment)	3.368	2	1.684	6.290	0.034
Discrepancy	1.606	6	0.268		
Sum of between groups	4.974	8			
Time of Measure	0.141	2	0.070	0.392	0.684
Time of Measure * Exp. Unit	0.484	4	0.121	0.674	0.623
Discrepancy	2.156	12	0.180		
Sum of within group	2.781	18			
Sum Total	**7.755**	**26**			

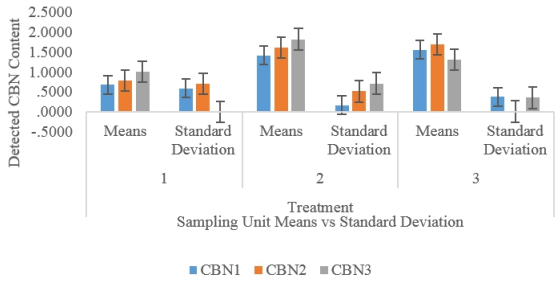

Figure 6: Observation on the measured values of detected CBN content (CBN1-3 measuring time-points) related to the sampling unit (each of 3 replicated beehives) means.

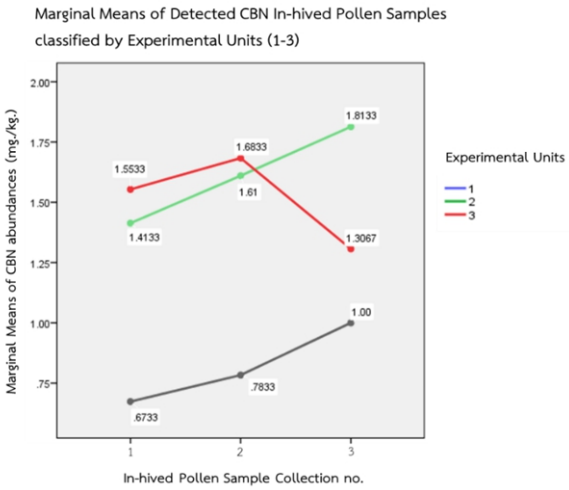

Figure 7: Means of detected Cannabinol (CBN) illustrated by treatment (experimental unit).

4. Conclusion, Discussion and Suggestion

In the conclusion, as the research results, there was a scientific proof that the unique compounds of Cannabinoids (CBD, THC and CBN), which are rich in *Cannabis sativa* L. (hemp plant), are transferred to bee products by means of raising *Apis mellifera* L. (honey bees) on the hemp plantation. This experimental results reiterated the theory of unique phytochemicals in plants, especially prolific flowers were transferred to and found in bee products [17], so called 'biological transformation'. The therapeutically bioactive potency of *Cannabis sativa* is apparent worldwide [18] since its content of unique cannabinoid constituents that are only found in cannabis plants also the demand of hemp products has been increasing, therefore, the industrial hemp plantations have been growing up [1] worldwide. Despite the fact that the decline of a number of bees in beekeeping industry in many countries are impacted from Colony Collapse Disorder (CCD) which is the unknown loss of bees. An expected cause is a floral resource scarcity during dearth period impacted from the climate changes and agricultural chemical uses etc. [6]. Therefore, mass flowering hemp crops can alternatively support honey bee population foraging [8, 9]. Since honey bees forage and collect hemp flower pollens as one of their individuals and colony's nutrients (protein), together with nectar (carbohydrate) discrete from plant flowers for their essentials to maintain colony healthy and propagation. This experiment was an alternative to cope with the impact. The hemp pollens were determined in the experiment rather than the plant resin, which one might recommend that there would be richer in cannabinoids contents. However, at this stage was designed as the prototype experiment, we needed to investigate which protocol is suitable choice to raise bees on hemp plantation in order to make them healthy and productive. Apart from the findings of the detection of CBD, THC and CBN in in-hive stored bee pollens, it also noted

that in the experimental methodology on different artificial supplements is separately fed to bees for investigation. We found that in the third treatment, even though bees are free foraging in opened hemp field, their choices of collecting other flower pollens are vastly, they also collect hemp pollens into hive as similar amounts as ones being confined in treatments with the netted hemp plantation. The foraging bees are preferable to collect massive hemp pollens for their food storages as the same findings as other researches [6, 8]. There was no sign on any negative impact to honey bees consuming Cannabinoids in all treatments during the experiment to support the previous study due to the lack of cannabinoid receptors in insects [10].

According to the findings, it would be useful in terms of business development in the future, especially for CBD which is high in *Cannabis sativa* L. (hemp), for example, CBD infused honey through the biological transformation. We suggest that this would be customized on demands for healthy food industry or even for Thai traditional and complementary medicine. Further research explorations in using bee raising protocol to produce bee products and long-terms impact to bees could be taken into consideration. Last, we would recommend to concerned authorities in considering the guidelines to control of *Cannabis sativa* L. strains with high contents of psychoactive THC and CBN potency to prevent any misconducting.

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