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Cannabinoids Bee Pollen: Presences of Psychoactive Compound Δ9-tetrahydrocannabinol (THC) and Cannabinol (CBN) in *Cannabis sativa* L. (Hemp) on The Characteristics of *Apis mellifera* L. Raising Protocol in Samoeng, Chiang Mai, Thailand

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Abstract:

Honey bees (Apis mellifera L.) are widely kept by the beekeeping industry since they are relatively gentle and calm easy to obtain, excellent foragers, moderate tendency to swarm, and good, compact brood pattern resulting in a strong workforce for collecting a good amount of nectar and pollen. While Δ9-tetrahydrocannabinol (THC) and Cannabinol (CBN) are psychoactive constituents. THC and CBN have been well recognizing as potent cannabinoids, unique phytochemical compounds encounter only in Cannabis sativa (hemp) plants. This experimental research aimed to investigate the presences of Δ9-tetrahydrocannabinol (THC) and Cannabinol (CBN) via a prototype of bee-raising protocol foraging in hemp cultivar to collect hemp pollens. Due to the lack of Endo-cannabinoid system (ECS) in bees that does not likely to negatively affect to them. Theoretically, in-hived stored hemp pollen shall embrace phytochemicals as being abundance in hemp plants, thru biological (biotic) extraction process. In the field experiment, seed production hemp plot in Samoeng, Chiang Mai was fully covered by mosquito net to confine honey bees during male flowering. The extraction of THC and CBN of in-hived stored pollen samples and their abundances were conducted by GC-MS technique at Central Laboratory (Chiang Mai Office). A repeated measures ANOVA model was conducted for statistical analysis. The result was presences of psychoactive compounds, THC and CBN in hemp plants to in-hived honey bee pollen as bee raising produce, significantly (P-value < 0.0009 and =0.034, respectively). However, their intensity were not much in nominal terms due to the Cannabis sativa strains were planted for fiber and seed production in this experiment and less THC and CBN intensities.

Keywords: *Cannabis sativa L. (Hemp), Δ9-tetrahydrocannabinol (THC), Cannabinol (CBN), Apis mellifera, GC-MS, Repeated Measures ANOVA*

I. INTRODUCTION

Honey bees (*Apis mellifera* L.) are widely kept by the beekeeping industry since then due to they are relatively gentle and calm easy to obtain, moderate to high cleaning behaviour, white capping in common, excellent foragers, moderate tendency to swarm, and good, compact brood pattern resulting in a strong workforce for collecting a good amount of nectar and pollen [1], [2]. Their characteristics considerably generate the good yields for honey bee produces [3].

Δ9-tetrahydrocannabinol (THC) and Cannabinol

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(CBN) are psychoactive compounds. They have been well recognizing as potent Cannabinoids, unique phytochemical constituents encounter only in *Cannabis sativa* (hemp) plants. Though isolation and synthesis of pure cannabinoids, including more potent synthetic, and the discovery of cannabinoid receptors and the Endo-cannabinoid System (ECS) in human's Central Nerve System (CNS) and immune cells [4]-[7] led to draw the interest in potential medical uses, internationally. This has also been stimulating by the existing claims of THC used for medicinal purposes. However, there are arguments or at least differences in opinion on crude THC and pure (synthetic) THC cannabinoids unless a proven in clinical trials [8]. Therefore, their unique characteristics and therapeutically bioactive potency would be vital in medicinal, healthcare purposes if one could manage to get this powerful phytochemical compound to honey bee produces in a symbiosis process.

Recent studies have documented the importance of hemp pollen in supporting a diverse community of honey bees during periods of floral resource scarcity [9], [10]. Mass flowering crops such hemp cultivar can support pollinator populations foraging [11], [12]. 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 [13]. However, the incorporation of novel hemp pollens into the diets of general bees has yet been shown to have detrimental effects on larval development [6]. Therefore, during the bee-foraging season would be potentially seen sparkling changes in potential hemp floral resources gaining in popularity in beekeeping industry.

The research aimed to investigate and identify THC and CBN in *Cannabis sativa* plant being transferred by means of honey bee raising protocol into in-hive stored pollens via 'cannabinoids biological/ biotic extraction' process. As a crucial improvement of Thailand beekeeping industry would extended related supply downstream industries i.e. healthcare or supplementary food or even Thai traditional medicine purposes as soon as the Government's endorsement on commercial hemp growing in Thailand in a near future.

II. METHODS

A. Field experiment method

The total of 9 hives of raising honey bees (*Apis mellifera* L.) were moved to 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) where elevation of 720 metre above mean sea level, average temperature of 18.8-29.6 degree Celsius, average air humidity of 53.6-95 percent (at mean of 74.3%) and rainfall average of 1,075.5 mm. and photoperiod of 11-13 hours daily. 6 Honey beehives were confined within mosquito net (mesh 16 threads per square inch) covering the hemp plot to ensure collected data of only hemp pollen from beehives and also protect bees from other predators during experimental period (Figure 1). The other 3 beehives were set up outside the mosquito net area as controlled experimental units.

All experimental beehives being raised in innovated beehive with monitoring system in order to reduce stress of bee individual and colony well-beings which lead to healthier and more efficient in foraging behaviour in return. Each group of 3 beehives were distinguished into 3 treatments (Ts) as T1- kept in netted hemp plot and in-hive drop-fed with diluted honey; T2- kept in netted hemp plot and in-hive drop-fed with diluted sugar syrup; and T3- kept outside netted hemp plot without any artificial feeding.

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

B. Sample collection process

The in-hive-stored Pollens, during the male flowering peak, were collected from brood frames

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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 beehive for sufficient laboratory investigation. Each of pollen vials was separately labelled and recorded by each beehive/ treatment for Laboratory test.

C. Laboratory

Gas Chromatography-Mass Spectrometry Method (GC-MS) [14]-[16]. was conducted by the certified Central Laboratory (Thailand) Co., Ltd. (Chiang Mai Branch): CLT for investigation and identification of THC and CBN abundance/ intensity of each samples.

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 x 30m x 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/CBN: RESTEX (34014) USA.

III. RESULTS

Laboratory results

It found that detected THC and CBN abundance/ intensity in all samples of treatment 1 (with honey fed during field research) were slightly lesser than those of treatment 2 and 3 (with diluted sugar fed and no feeding during field research, respectively). These results need for further investigation in terms of impact factors. However, this was beyond of scopes of work in this experiment, but as it was concerned then need to be pay attention in next stage.

Table 1 THC and CBN intensity/ abundances being detected from in-hive stored pollen samples.

		Measure no. by each collection			
Experimental Unit no.1 (Treatment # 1)	Hive #1	**CM62/10544-001**	**T1H1-1**	**T1H1-2**	**T1H1-3**
		THC (RT 18.79 min.)	8.69 0.09%	16.92 0.17%	14.56 0.15%
		CBN (RT 19.28 min.)	< 1.00 NA	1.35 0.01%	< 1.00 NA
	Hive #2	**CM62/10544-002**	**T1H2-1**	**T1H2-2**	**T1H2-3**
		THC (RT 18.79 min.)	17.04 0.17%	12.19 0.12%	< 1.00 NA
		CBN (RT 19.28 min.)	1.02 0.01%	ND ND	< 1.00 NA
	Hive #3	**CM62/10544-003**	**T1H3-1**	**T1H3-2**	**T1H3-3**
		THC (RT 18.79 min.)	17.46 0.17%	14.41 0.14%	17.05 0.17%
		CBN (RT 19.28 min.)	ND ND	< 1.00 NA	< 1.00 NA

Remark: THC and CBN unit is mg/kg

Table 1 THC and CBN intensity/ abundances being detected from in-hive stored pollen samples (Cont.)

		Measure no. by each collection			
Experimental Unit no.2 (Treatment # 2)	Hive #1	**CM62/10544-001**	**T2H1-1**	**T2H1-2**	**T2H1-3**
		THC (RT 18.79 min.)	8.62 0.09%	7.75 0.08%	2.75 0.03%
		CBN (RT 19.28 min.)	1.6 0.02%	2 0.02%	2.26 0.02%
	Hive #2	**CM62/10544-002**	**T2H2-1**	**T2H2-2**	**T2H2-3**
		THC (RT 18.79 min.)	3.32 0.03%	1.05 0.01%	< 1.00 NA
		CBN (RT 19.28 min.)	1.34 0.01%	1.8 0.02%	2.18 0.02%
	Hive #3	**CM62/10544-003**	**T2H3-1**	**T2H3-2**	**T2H3-3**
		THC (RT 18.79 min.)	1.72 0.02%	6.95 0.07%	7.53 0.08%
		CBN (RT 19.28 min.)	1.3 0.01%	1.03 0.01%	1 0.01%

Remark: THC and CBN unit is mg/kg

Table 1 THC and CBN intensity/ abundances being detected from in-hive stored pollen samples (Cont.)

		Measure no. by each collection			
Experimental Unit no.3 (Treatment # 3)	Hive #1	**CM62/10544-001**	**T3H1-1**	**T3H1-2**	**T3H1-3**
		THC (RT 18.79 min.)	1.38 0.01%	1.08 0.01%	< 1.00 NA
		CBN (RT 19.28 min.)	1.13 0.01%	1.68 0.02%	1.69 0.02%
	Hive #2	**CM62/10544-002**	**T3H2-1**	**T3H2-2**	**T3H2-3**
		THC (RT 18.79 min.)	4.5 0.05%	3.51 0.04%	ND ND
		CBN (RT 19.28 min.)	1.84 0.02%	1.68 0.02%	1.23 0.01%
	Hive #3	**CM62/10544-003**	**T3H3-1**	**T3H3-2**	**T3H3-3**
		THC (RT 18.79 min.)	1.85 0.02%	ND ND	ND ND
		CBN (RT 19.28 min.)	1.69 0.02%	1.69 0.02%	< 1.00 NA

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Limit of Quantification-LOQ	1.00	1.00	1.00
Limit of Detection-LOD	0.50	0.50	0.50

Remark: THC and CBN unit, LOQ, and LOD are mg/kg

Statistical analysis

Using the Repeated measures ANOVA model by paralleling means across measure variables that were based on repeated observations. As seen comparison and analysis below.

Table 2 Detected Cannabidiol (CBD) abundance (mg/kg) classified by the experimental units

Experimental Unit (Treatment#)	Sampling Unit (Beehive#)	Measure 1	Measure 2	Measure 3
1	1	8.69	16.92	14.56
	2	17.04	12.19	16.17
	3	17.46	14.41	17.05
2	1	8.62	7.75	2.75
	2	3.32	1.05	1.00
	3	1.72	6.95	7.53
3	1	1.38	1.08	1.00
	2	4.50	3.51	1.00
	3	1.85	1.00	1.00

Analysis of Variance of detected Δ9-tetrahydrocannabinol (THC) abundance/ intensity

Table 3 Analysis of Variance of detected Cannabidiol (CBD) abundance/ intensity

Sources of Variation	Sum of Squares	Degrees of Freedom
Experimental Unit	865.055	2
Discrepancy	54.307	6
Sum of between Groups	919.36	8
Time of Measure	0.528	2
Time of Measure * Exp. Unit	10.916	4
Discrepancy	94.467	12
Sum of Within Group	105.91	18
Sum Total	**1,025.27**	**26**

Table 3 Analysis of Variance of detected Cannabidiol (CBD) abundance/ intensity (Cont.)

Sources of Variation	Root Means Square (RMF)	F	P-value
Experimental Unit	432.528	47.787	<0.0009
Discrepancy	9.051		
Sum of between Groups			
Time of Measure	0.264	0.034	0.967
Time of Measure * Exp. Unit	2.729	0.347	0.841
Discrepancy	7.872		

From the test, it 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 is given, the findings were that none of means differences of experimental unit 2 and 3 is found with lesser means than experimental unit 1.

Where $F_5 = 0.034$ It could be concluded that none of different means is 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 measure is found with experimental units P-value = 0.841.

It could be interpreted that among 3 different treatments, as above statistical result shown; among the group of treatments, each treatment has different means of detected THC which is significant (P-value < 0.0009). While none of different means within each group shown no statistically significant difference (by each measure and within each treatment P-value > 0.05). Therefore, measure data within a group of treatments are the same means of detected THC. When comparing average means among all treatments; detected THC abundance/ intensity of treatment 2 (with diluted syrup in-hive fed and detained bees within netted hemp cultivar) and treatment 3 (without feeding, with free foraging bees in opened hemp cultivar) are lesser than treatment 1 (with diluted honey in-hive fed and detained bees within netted hemp cultivar). Naturally, hemp plants are less potent of THC and also since pollen samples are in fresh forms (yet in neutral forms) which are preserved by bees mixing with honey or artificial honey.

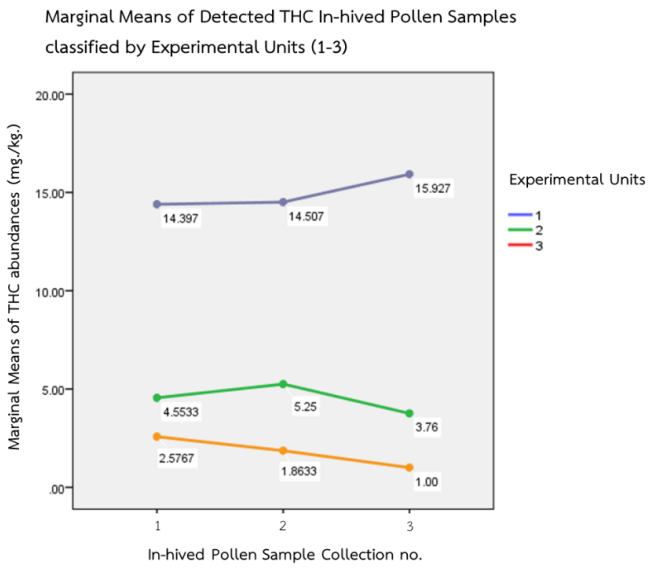

Figure 2 Means of detected Δ9-tetrahydrocannabinol (THC) illustrated by experimental units

Detected Cannabinol (CBN) abundance/ intensity (mg/kg) by the experimental unit classification

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Table 4 Detected Cannabinol (CBN) abundance/ intensity (mg/kg) classified by the experimental units

Experimental Unit (Treatment#)	Sampling Unit (Beehive#)	Measure 1	Measure 2	Measure 3
1	1	1.00	1.35	1.00
	2	1.02	0.00	1.00
	3	0.00	1.00	1.00
2	1	1.60	2.00	2.26
	2	1.34	1.80	2.18
	3	1.30	1.03	1.00
3	1	1.13	1.68	1.69
	2	1.84	1.68	1.23
	3	1.69	1.69	1.00

Analysis of Variance of detected Cannabinol (CBN) abundance/ intensity

Table 5 Analysis of Variance of detected Cannabinol (CBN) abundance/ intensity

Sources of Variation	Sum of Squares	Degrees of Freedom
Experimental Unit	3.368	2
Discrepancy	1.606	6
Sum of between Groups	4.974	8
Time of Measure	0.141	2
Time of Measure * Exp. Unit	0.484	4
Discrepancy	2.156	12
Sum of Within Group	2.781	18
Sum Total	7.755	26

Table 5 Analysis of Variance of detected Cannabinol (CBN) abundance/ intensity (Cont.)

Sources of Variation	Root Means Square (RMF)	F	P-value
Experimental Unit	1.684	6.290	0.034
Discrepancy	0.268		
Sum of between Groups			
Time of Measure	0.070	0.392	0.684
Time of Measure * Exp. Unit	0.121	0.674	0.623
Discrepancy	0.180		

From the test, it 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 is given, the findings were that Experimental units 2 and 3: means do not differ. Experimental units 1 and 3: means do not differ. Experimental unit 2: means is greater than those of experimental unit 1

Where $F_8 = 0.392$ It could be concluded that none of different means is found in each measure of CBN P-value = 0.684.

Where $F_9 = 0.674$ It could be concluded that none of different interaction within measure is found with experimental units P-value = 0.623.

It could be interpreted that among 3 different treatments, as above statistical result shown; among the group of treatments, each treatment has different means of detected CBN which is significant (P-value = 0.034). While none of different means within each group shown insignificant (by each measure and within each treatment P-value > 0.05). When comparing average means among all treatments; detected CBN abundance/ intensity of treatment 2 (with diluted syrup in-hive fed and detained bees within netted hemp cultivar) is greater than treatment 1 (with diluted honey in-hive fed and detained bees within netted hemp cultivar). While average means of detected CBN between treatment 1 and treatment 2 as well as those between treatment 1 and treatment 3 are not different.

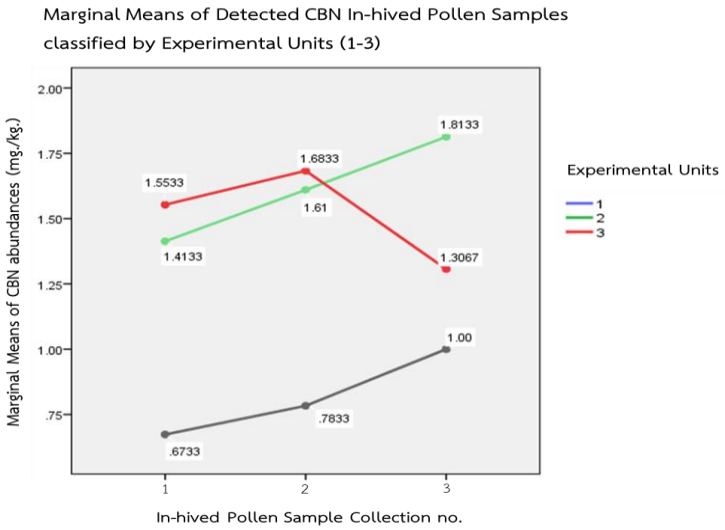

Figure 3 Means of detected Cannabinol (CBN) illustrated by experimental units

CONCLUSION

The theory on phytochemicals transfer from plants, especially prolific flowers to bee. This was also considered as a biological (biotic) extraction in natural rather than chemicals used by laboratory or industry. Since this first found experiment on how to manage bees to verify the natural extraction of cannabinoids, which are rich in *Cannabis sativa* L. (industrial hemp) depends upon their strains and landraces. Since *cannabis sativa* L. (hemp) plants

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produce large amounts of pollen that are attractive to bees, thus to manage bees was a crucial experiment. With some techniques deployed in order to calm bees for their utmost efficiency while induce stress to hemp plants expected to optimize level of outputs both from bees and plants. During the time of seasonal dearth of natural/ crop foods availability in apiaries, commercially grow hemps should be one of alternatives to develop model of beekeeping industry of Thailand which have been struggling with climate changes vastly reflect to commercial crop plants and cultivation practices and inefficiently controllable uses of pesticides and herbicides. This experiment could be an inspiration for further exploration of studies on negative impacts on bee reproductive system even through there was a study on a lack of Endo-cannabinoid system (ECS) in bees that will not be unsafely affect their brains as in human. Since *Cannabis sativa* L. strains are variety upon their richness of cannabinoids and other constituents concerning health benefits which could be designed and manage in terms of individual and business to evaluate techniques and return on investment according to their goals in future. Since this experiment, using a symbiosis approach as experimental method, in the other word, interdisciplinary study approach on bee-raising management and *Cannabis sativa* L. plantation. It could be observed and learnt from this novel experiment that at present, techniques have been adopting for extraction either for medical, recreation or other purposes for food supplements generally using the biosynthetic extraction methods. While a biological (biotic) extraction for natural produces could be adaptably used in other sectors i.e. nutritional supplements in Thai Traditional and complementary medicine and also for green agroindustry purposes. The suitable plant strains and bee species in Thailand could be necessities for further explorations.

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