Research Article

Development of a plant-produced recombinant monoclonal antibody against Δ-9-tetrahydrocannabinol (Δ9-THC) for immunoassay application

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ABSTRACT

Δ-9-tetrahydrocannabinol (Δ9-THC) is mainly a psychoactive compound in the cannabis plant. The immunoassay, an alternative method to HPLC and GC, can be used to analyze and measure Δ9-THC. This method provides high sensitivity and specificity by using antibodies specific to the desired substances. Currently, plants provide several benefits over traditional expression platforms to produce recombinant antibodies, such as lower production costs and scalability. Therefore, this study aims to produce a recombinant anti-Δ9-THC monoclonal antibody (mAb) with transient expression using *N. benthamiana*. The highest expression level of the plant-produced mAb was estimated to be 0.33 ug/g leaf fresh weight. Our results demonstrate that the antibody provided in vitro affinity binding related to Δ9-THC and the metabolites of Δ9-THC, such as cannabinol (CBN). Moreover, the antibody also showed binding efficiency with Δ9-THC in cannabis extract. Moreover, plant-produced mAbs provide efficiency against Δ9-THC and can be applied for further immunoassay applications.

1. Introduction

Marijuana and hemp are the same species, *Cannabis sativa* L. More specifically, marijuana is *C. sativa* subsp. *Indica*, and hemp is *C. sativa* subsp. *sativa*. They are flower-bearing plants and belong to the Cannabaceae family. [1]. Hemp is mainly used for industrial purposes such as cosmetics and foods [2, 3], while marijuana has a long history of being used as a medicine to reduce pain, treat neurodegeneration and neuroprotection and multiple sclerosis, control epilepsy and provide anticancer effects [4–6]. The main cannabinoids in cannabis are cannabidiol (CBD), cannabinol (CBN), and Δ−9-tetrahydrocannabinol (Δ9-THC). Only THC is a psychoactive compound [7]. In addition, many drug abusers are addicted to consume *cannabis* to obtain a high sensation. This narcotic can affect drug addicts in terms of emotional instability, body movement disorder, cognitive functioning, etc. For long periods of time, mental effects will occur, such as a dependence syndrome and schizophrenia [8–11].

In Thailand, the maximum permitted concentration of Δ9-THC in cannabis-based food products should not exceed 0.2% of the dry weight of the product (Thailand Food & Drug Administration, Notification No. 425). Additionally, the amount of Δ9-THC in food products available in the market derived from cannabis can be present at a low concentration (1 ppm). Thus, it is necessary to develop new analytical techniques that are sufficiently sensitive to detect the quantity specified for its legal use.

To date, many approaches have been applied for Δ9-THC detection, such as thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography and mass spectrometry (HPLC–MS) [12, 13]. Although these approaches provide a highly accurate result, they were conducted by a complicated protocol and required specialists, and they have limitations for detection and quantification. Thus, an alternative technique for detection with rapid and convenient testing was conducted in this study.

Immunoassays are methods that have been developed to detect specific analytes. This type of analysis is fast, simple, highly sensitive and specific. Moreover, immunoassays have a high throughput capacity and can be applied for the on-site analysis of many samples [14–16]. Currently, immunoassays are widely used in various applications such as diagnostic reagents for infectious diseases and detection reagents for

low-molecular-weight hapten targets [17]. This bioanalytical method can be conducted by enzyme-linked immunosorbent assay (ELISA) using the reaction of the analytes or antigen binding to specific antibodies [18].

For cost-effective immunoassay development, the bacterial expression system is a commonly used platform to produce the recombinant proteins, antibody and antigen. However, the bacterial system presented limitations in the recombinant antibody production due to a lack of post-translational modifications (PTMs) [19–21]. Nevertheless, there are many expression systems to produce recombinant protein, and one of the attractive platforms is a mammalian cell. For complex protein production, mammalian cells are predominant in the production of various approved recombinant proteins, including vaccine enzymes and antibodies [22, 23]. However, this process requires high operating costs and limited scalability [24–27].

Recently, a plant-based expression system has become an alternative production platform and provides several advantages among various expression platforms, including a lower production cost, scalability, rapid growth rate and capability to perform PTMs. [24, 28-37]. According to previous studies, the plant-expression system is a cost-effective platform for producing recombinant proteins for many immunoassay applications. Therefore, this study aimed to produce the anti-Δ9-THC mAb in *N. benthamiana* using a transient expression system and investigate the binding properties between plant-produced mAb and Δ9-THC, which is the main psychoactive compound contained in the *cannabis* plant [38]. Overall, this study provides a proof of principle for the production of anti-Δ9-THC mAb in a plant expression system, which demonstrates the binding potential to Δ9-THC, and it can be applied for further immunoassay applications and testing on commercial products.

2. Materials and methods

2.1. Plant expression vector construction

The amino acid sequences of variable regions (V_H and V_L) of anti-Δ9-THC T3 Fab fragment (PDB accession number: 3LS4_H and 3LS4_L) and human IgG1 constant regions were previously reported [39, 40]. The V_H and V_L amino acid sequences were codon-optimized for *N. benthamiana* expression and the optimized gene sequences were synthesized (Biomatik, Canada). The V_H and V_L genes were digested with *BsaI*/*NheI* and *BsaI*/*AflII* restriction enzyme, respectively, and the digested products were gel extracted and purified. The gel-extracted product of V_H was then ligated with constant (C_H1–C_H2–C_H3) regions of the heavy chain (HC) in a geminiviral expression vector (pBY3R) [41] using T4 DNA ligase (New England Biolabs, Hitchin, UK). For the light chain (LC), the V_L genes was ligated to constant (C_L) regions of the light chain (LC) in pBY3R. The barley alpha-amylase signal peptide was added to the amino terminus (N-terminus) [42], and SEKDEL was added to the carboxyl terminus (C-terminus) of both the heavy chain (HC) and light chain (LC). The pBY3R-anti-Δ9-THC HC and LC vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101 *via* electroporation. Polymerase chain reaction (PCR) technique with gene-specific forward and reverse primers was used for confirming the positive *Agrobacterium* clones. Taq DNA polymerase (Vivantis Technologies, Malaysia) was used for amplification. (The PCR cycling conditions: initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30–60 s and a final extension at 72 °C for 5 min), and then the products were separated on 1% agarose gel. *A. tumefaciens* harboring pBY3R-anti-Δ9-THC HC and LC vectors were used for further experiments.

2.2. Transient expression of anti-Δ9-THC mAb in N. benthamiana

The positive *A. tumefaciens* harboring pBY3R-anti-Δ9-THC HC and LC vectors was cultured on selective Luria–Bertani (LB) medium containing 50 μg/ml rifampicin, gentamicin and kanamycin and then shaking incubated at 28 °C at 200 rpm for overnight. The bacterial cell was centrifuged at 4000 x g for 10 min and the pellet was resuspended with infiltration buffer (10 mM 2-[N-*morpholino*]ethanesulfonic acid (MES) and 10 mM MgSO4, pH 5.5) to reach an optical density of 600 nm (OD_{600}) of 0.2. Each cell suspension containing either pBY3R-anti-Δ9-THC HC and LC was mixed equally and co-infiltrated into wild-type *N. benthamiana* by syringe infiltration. The infiltrated plants were incubated at 28 °C with a 16-h light/8-h dark cycle and harvested at 2-, 4-, 6- and 8-days post infiltration (dpi). To quantify the plant-produced mAb, ELISA was performed. Then, vacuum infiltration was performed for mAb large-scale production.

2.3. Anti-Δ9-THC mAb extraction and expression level quantification

The infiltrated leaves were harvested and extracted with 1x phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4). To remove the cell debris, the crude extract was centrifuged at 26,000 g for 40 min at 4 °C. Then, sandwich ELISA was performed to quantify the plant-produced mAb expression level. 50 μl anti-human IgG-Fc fragment specific (Abcam, United Kingdom) at 1:1000 dilution in 1x PBS (pH 7.4) was captured into 96-well ELISA plates (Greiner Bio One GmbH, Austria) for overnight. Then, the plates were blocked with 200 μl 5% skim milk in 1x PBS. and coated with human IgG1 kappa isotype antibody (Abcam, United Kingdom) or plant-produced mAb at varying dilutions in 1x PBS and incubated at 37 °C for 2 h. Each sample was loaded in triplicate wells. After that, HRP-conjugated anti-human kappa antibody (Southern Biotech, United States) with a dilution of 1:1000 in 1x PBS was incubated into the plate for 1 h at 37 °C. SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (Promega, United States) was added followed by 1 M H_2SO_4 to terminate the reaction, and the absorbance was measured using a microplate reader at an optical density of 450 nm (OD_{450}). 1x PBST (1xPBS containing 0.05% Tween-20) was washed three times in each step.

2.4. Anti-Δ9-THC mAb purification

The vacuum-infiltrated leaves were harvested and extracted with 1xPBS extraction buffer. Before loading into a protein A affinity chromatography column, the crude extract was clarified by centrifugation, and the supernatant was filtered through a 0.45 micron filter (Millipore Sigma, United States). Amintra® Protein A Resin (Expedeon, United Kingdom) was packed into the column and then equilibrated with 1x PBS (pH 7.4). After loading the clarified crude extracts, the column was washed with 1x PBS (pH 7.4). The protein was eluted with elution buffer (0.1 M glycine, pH 2.7) and neutralized with 1.5 M Tris–HCl (pH 8.8). Then, the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or subjected to western blotting.

2.5. SDS-PAGE and western blotting

To determine the purity of the plant-produced mAb, SDS-PAGE and western blotting were performed. The samples were mixed with loading buffer (125 mM Tris–HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue) under non-reducing condition and then denatured at 95 °C for 5 min. The samples were separated on 6%−10% polyacrylamide gels. For reducing conditions, the sample was mixed with loading buffer containing 22% β-mercaptoethanol, denatured at 95 °C for 5 min and then separated on 15% polyacrylamide gels. Human IgG1 kappa isotype antibody was used as a positive control. After that, the gels were stained with Coomassie brilliant blue (Appli-Chem, Germany) to visualize the bands. According to western blot analysis, the polyacrylamide gel containing separated protein was electro-transferred to a nitrocellulose membrane (Bio-Rad, United States). After blocking with 5% skim milk in 1x PBS (pH 7.4), the

membranes were incubated either with HRP-conjugated anti-human gamma antibody (The Binding Site, United Kingdom) or HRP-conjugated anti-human kappa antibody (Southern Biotech, United States) at a 1:5000 dilution in 3% skim milk prepared in 1x PBS (pH 7.4). Then, the membrane was developed with Amersham ECL prime western blotting detection reagent (GE Healthcare, United Kingdom). 1x PBST (1xPBS containing 0.05% Tween-20) was washed three times in each step.

2.6. Binding efficiency of plant-produced mAb to Δ9-THC

2.6.1. Preparation of standard solution

Stock solution of reference standards including Cannabidiol (CBD), cannabinol (CBN) and Delta-9-tetrahydrocannabinol (Δ9-THC), cannabidiolic acid (CBDA) (Cayman chemical, United States), Δ 9-tetrahydrocannabinolic acid A (THCA-A) (THC GmbH The Health Concept, Germany), daidzein, caffeine and gallic acid (Sigma-Aldrich®, United States) was prepared by dissolving in DMSO at a concentration of 1 mg/mL and was further diluted with the same solvent to obtain working solutions (10 μg/mL). All stock and working solutions were stored at −20 °C till further use. Dried hemp including inflorescences, leaf, stem, and root were from a single strain which was cultivated in the close system at the Faculty of Dentistry, Chulalongkorn University. Dried marijuana was granted by Office of the Narcotics Control Board (ONCB, Bangkok, Thailand).

2.6.2. Competitive ELISA for plant-produced mAb binding to Δ9-THC

To investigate the binding activity, competitive ELISA was performed. 96-well ELISA plates (Greiner Bio One GmbH, Austria) were coated with 500 ng of THC–BSA conjugate (Fitzgerald Industries International, Inc., Massachusetts) in 50 μl. Then, the plate blocked with 200 μl 5% skim milk in 1x PBS at 37 °C for 2 h. The samples (200 μl) containing 0.2 μg/ml of plant-produced anti- Δ9-THC mAb with reference standards or *Cannabis* crude extracts with various concentration were incubated in 1.5 microcentrifuge tubes for 1 h at room temperature. After that, 50 μl of samples was pipetted into THC–BSA-coated wells. Each sample was loaded in triplicate wells. HRP-conjugated anti-human kappa antibody with a dilution of 1:1000 in 1x PBS was incubated into the plate for 1 h at 37 °C. Then, SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (Promega, United States) was added followed by 1 M H_2SO_4 to terminate the reaction, and the absorbance was measured using a microplate reader at an optical density of 450 nm (OD_{450}). 1x PBST (1xPBS containing 0.05% Tween-20) was washed three times in each step.

2.7. Qualification of cannabinoids and quantitation of Δ9-THC in C. sativa

2.7.1. Preparation of standard solution

Stock solution of CBD, CBN and Δ9-THC standard was prepared by dissolving in methanol at a concentration of 1 mg/mL and was further diluted with the same solvent to obtain working solutions (100 μg/mL), while working solutions of CBG, CBDA, THCA standards were prepared from the 1 mg/mL certified standard solutions by diluting with acetonitrile (Merck, United States). All stock and working solutions were stored at −20 °C till further use.

2.7.2. Sample preparation

Dried hemp samples including inflorescences, leaf, stem, and root were from a single strain which was cultivated in the close system at the Faculty of Dentistry, Chulalongkorn University. All dried hemp samples were ground into fine powder. One gram of the powder was sonicated in 10 mL of methanol; chloroform (9:1, v/v) for 30 min and further centrifuged at 5000 rpm for 5 min. The supernatants were evaporated and prepared at concentration 5 mg/mL in methanol: chloroform (9:1, v/v). Then the solutions were filtered and used as a test solution.

For the marijuana sample obtained from ONCB, it was subjected to supercritical fluid extraction (SFE). SFE condition was the following: using $scCO_2$ as solvent at 67.5 °C, under a pressure of 250 bar at a flow rate of 20 Hz. The total time of extraction was 4.5 hours. Then the $scCO_2$-extract was winterized by dissolving with ethanol and further homogenized using GT sonic-D27 (GT sonic, China) for 10 min. The sample was incubated at −20 °C for overnight and filtered by Whatman no.1 filter paper for further use. Then the solution was evaporated. All solutions were prepared at concentration 1 mg/mL in methanol: chloroform (9:1, v/v), filtered with a 0.2 μm PTFE filter and used as the test solution.

2.7.3. HPLC analysis

The HPLC method as previously described[1] was followed with slight modifications. The analyses were carried out in an Agilent 1260 Infinity II HPLC system (Agilent Technologies, USA) including a flexible pump, an autosampler, a thermostated column compartment and a diode array detector (DAD) WR. Chromatographic separation was performed on an EC—C18 guard column (3.0 × 5 mm, 2.7 μm, InfinityLab Poroshell 120, Agilent, Country) with thermostated at 35 °C using a gradient of 0.1% formic acid in water (A) and acetonitrile (B) as the mobile phase. The gradient elution was performed at flow rate of 0.5 mL/min following 0–15.0 min; 70–80% B, 15.0–15.1 min; 80–95% B, 15.1–18.0 min; 95% B, 18.0–18.1 min; 95–70% B, 18.1–25.0 min; 70% B. The injection volume of sample and standard used was 5 μl and the peaks obtained monitored at a wavelength of 228 nm.

The total amount of Δ9-THC was calculated as the sum of the Δ9-THC content, and THCA content multiplied by a conversion factor (0.877) following American Herbal Pharmacopeia (AHP)[2]. The content of THCA and Δ9-THC in samples were quantified from a calibration curve prepared by serial dilution of stock solution of Δ9-THC standards in the range of 6.25–150 and 6.25–250 μg/mL in triplicates, respectively. The equation of the calibration curve ($y = mx + b$) was obtained by plotting the peak area (y) versus concentration (x). Linearity was acceptable with the correlation coefficient (r^2) greater than 0.995. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using the following formula: LOD = $(3.3 \times \sigma)/m$ and LOQ = $(10 \times \sigma)/m$, respectively. In the equation, the σ stands for the residual standard deviation of the calibration curve, and m is the slope of the calibration curve.

2.8. Statistical analysis

The data were analyzed from three individual experiments and presented as mean ± standard error of mean (SEM). The differences between means of the individual groups were analyzed using one-way analysis of variance (ANOVA) via GraphPad Prism 9.1 software (San Diego, CA, USA). p-value < 0.05 was considered as statistical significance.

3. Results

3.1. Recombinant anti- Δ9-THC mAb expression in N. benthamiana

To express a recombinant anti-Δ9-THC mAb, the nucleotide sequences of V_H and V_L of the anti-Δ9-THC T3 Fab fragment and IgG1 constant regions were modified with *N. benthamiana*-optimized codons. The genes were cloned into a geminiviral expression vector (pBY3R) (Fig. 1a) and subsequently electroporated into *A. tumefaciens* strain GV3101. Syringe infiltration was performed to co-infiltrate wild-type *N. benthamiana* leaves with *A. tumefaciens* harboring pBY3R-anti-Δ9-THC HC and LC to produce assembled antibodies, including 2HC and 2 LC (Fig. 1b). After the incubation, the infiltrated leaves showed strong necrosis, which was related to the days post-infiltration (dpi) (Fig. 2a). The time course experiment demonstrated that the expression level was shown on day 2 after infiltration and reached the highest expression
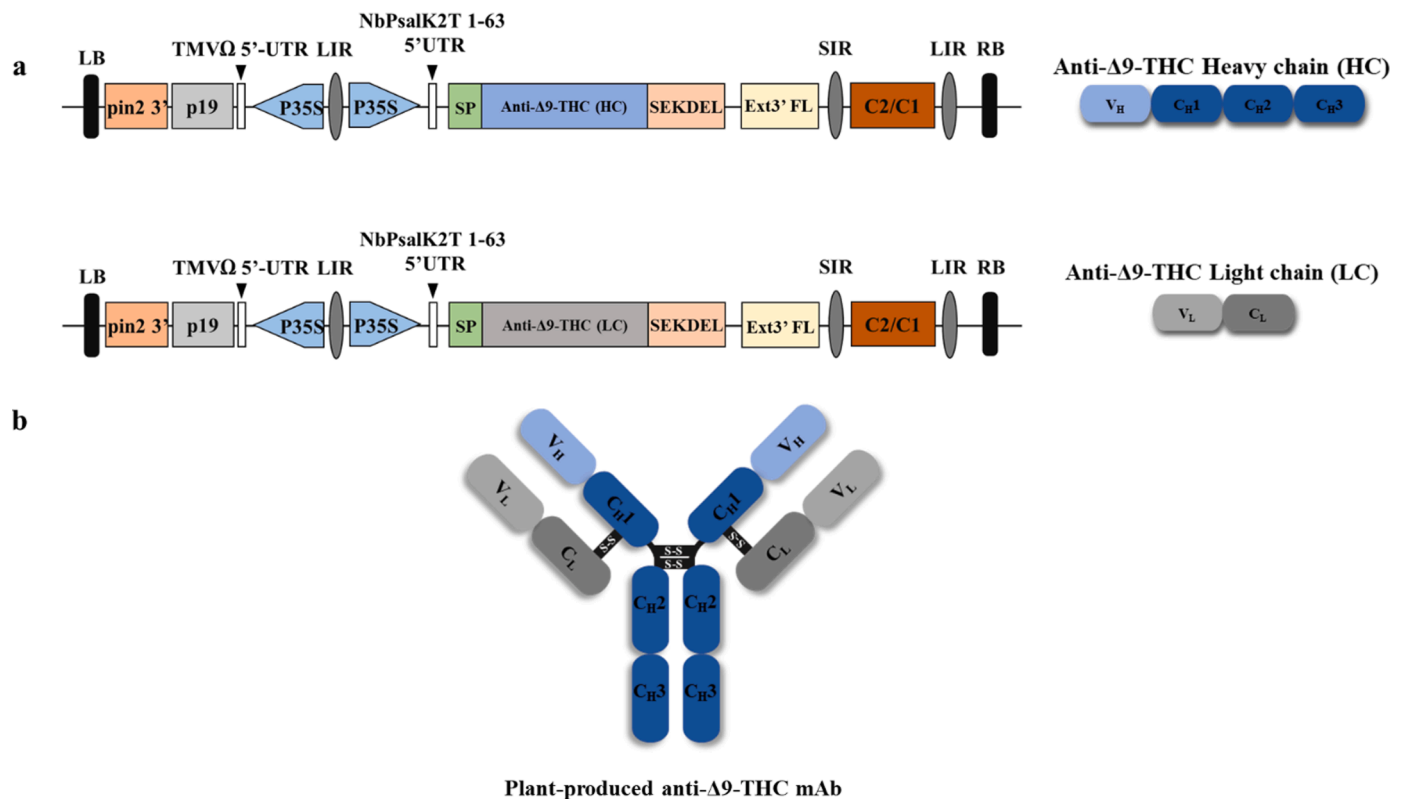
Fig. 1. Schematic representation of plant-produced anti- Δ9-THC mAb plasmid construction (a) The geminiviral expression vector (pBY3R), P35S, Cauliflower mosaic virus (CaMV) 35S promoter; NbPsaK2T 5′-UTR, 5′ untranslated region; SP, Barley alpha-amylase signal peptide; anti-THC mAb gene: anti-(-)-Δ 9-tetrahydrocannabinol (THC) monoclonal antibody; HC and LC, heavy and light chains of the antibody; SEKDEL, C-terminal endoplasmic reticulum (ER) retention signal peptide; Ext3' FL, 3′ full length of the tobacco extension gene; C2/C1, C1 and C2 gene of Bean Yellow Dwarf virus (BeYDV) encoding replication initiation protein (Rep) and RepA; LIR, long intergenic region of BeYDV genome; SIR, short intergenic region of BeYDV genome; TMVΩ 5′-UTR, tobacco mosaic virus Ω 5′ untranslated region; p19, p19 gene from Tomato Bushy Stunt virus (TBSV); LB and RB, left and right borders of the *Agrobacterium.* (b) Schematic and structural elements of assembled plant-produced anti-THC mAb.

level of plant-produced mAb on day 4 post infiltration (up to 0.33 μg/g leaf fresh weight) (Fig. 2b). However, the expression of plant-produced mAb was not observed at 6–8 dpi.

3.2. Recombinant anti- Δ9-THC mAb purification

Cultures of *A. tumefaciens* harboring pBY3R-anti-Δ9-THC HC and LC were diluted with infiltration buffer to reach an OD_{600} of 0.2. The plants were vacuum infiltrated; then, the infiltrated leaves were harvested at 4 dpi. After extraction with 1x PBS extraction buffer, the crude extract was purified using protein A affinity column chromatography. SDS–PAGE and western blotting were performed to analyze the purified plant-produced mAb. The SDS–PAGE gel of the purified plant-produced mAb was stained with Coomassie brilliant blue stain to visualize the separated protein bands. The expected band at 150 kDa was observed in the SDS–PAGE-stained gels under nonreducing conditions, which was related to the assembled antibody in a tetrameric form. According to the reducing conditions, the protein bands were visualized at approximately 50 and 25 kDa, which correspond to the HC and LC of the antibody, respectively, similar to the human IgG1 kappa isotype antibody (Fig. 3a and d, Lanes 2 and (+)). This observation suggests that protein A affinity chromatography can purify the plant-produced mAb from crude extracts (Fig. 3a and d, Lanes 1 and 2).

Western blotting was performed with anti-human gamma-HRP and anti-human kappa-HRP antibodies. Under nonreducing conditions, the protein band was detected at molecular sizes of approximately 150 kDa (Fig. 3b and c, Lanes 3 and 4), while the expected sizes at 50 and 25 kDa were observed in the SDS–PAGE-stained gels (Fig. 3e and f, Lanes 3 and 4). These results indicate that the co-infiltration of genes encoding HC

and LC produced the assembled anti-Δ9-THC mAb in *N. benthamiana* leaves and could be purified by protein A affinity chromatography.

### 3.3. Binding properties of plant-produced mAb to Δ9-THC

To determine the binding activity and specificity of plant-produced mAb to Δ9-THC, the competitive ELISA was performed by incubating the plant-produced mAb with reference standards in the concentration range of 0.625–10 μg/ml. The bound antibody was detected using HRP-conjugated anti-human kappa antibody. The plant-produced mAb provided higher binding affinity to Δ9-THC than CBN, which is the nonenzymatic oxidation byproduct of Δ9-THC [43]. In contrast, the plant-produced mAb showed no binding activity to other cannabinoid reference standards, including CBD, THCA and CBDA. Binding signals were also not observed for isoflavonoid compounds, including daidzein; alkaloid compounds, including caffeine; and phenolic compounds, including gallic acid. The results show that plant-produced mAb had specific binding properties to Δ9-THC and its metabolites (Fig. 4a). The crude extracts from hemp, including leaves, roots, stems, inflorescences, and marijuana, from ONCB in the concentration range of 0.3125–1 mg/ml were mixed with the plant-produced mAb. The binding properties of plant-produced mAb to Δ9-THC in ONCB, which was used as a positive control, were higher than the crude extracts from inflorescences and leaves, respectively. In contrast, the plant-produced mAb provided no binding efficiency on the stem and root extracts comparable to *Centella asiatica* extracts (as a negative control) (Fig. 4b). The results demonstrate that the plant-produced anti-Δ9-THC mAb exhibited specific binding to Δ9-THC and could detect Δ9-THC in *Cannabis* extracts.
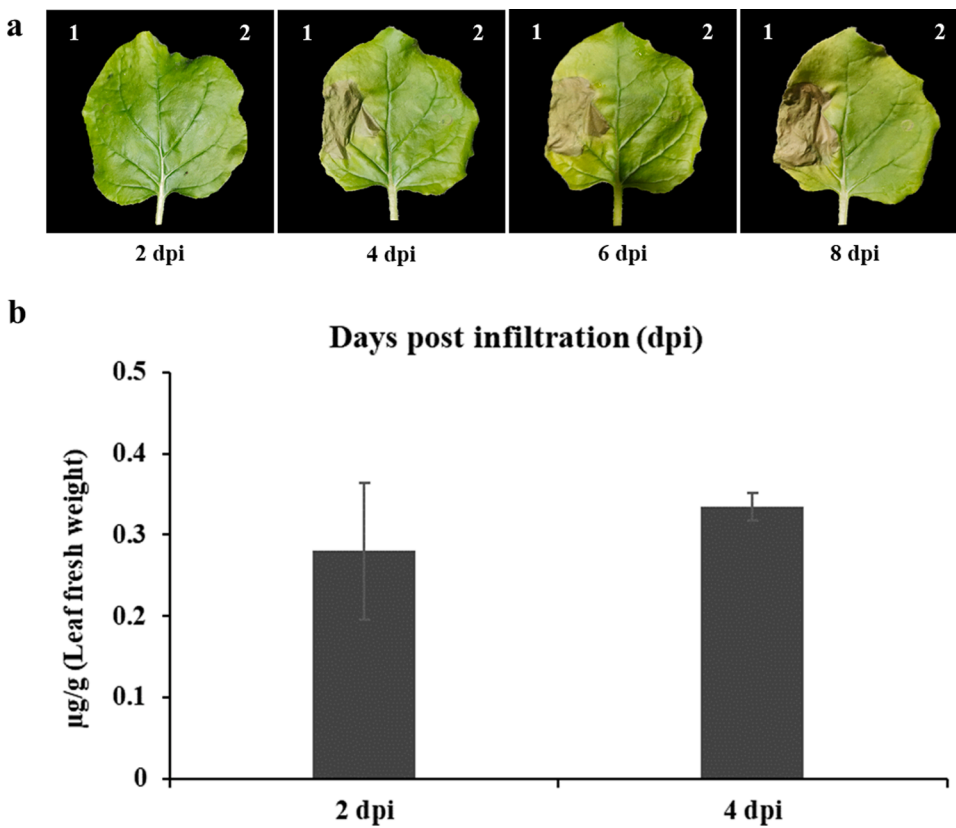
Fig. 2. Transient expression level of plant-produced anti- Δ9-THC mAb. (a) Infiltrated leaves phenotype during 2-, 4-, 6- and 8-days post infiltration (dpi). (1) Co-infiltrated *Nicotiana benthamiana* leaves with *A. tumefaciens* containing pBY3R-anti- Δ9-THC HC + LC and (2) *A. tumefaciens* without an expression vector. (b) Co-infiltrated *N. benthamiana* leaves were harvested at 2 and 4 dpi from 3 individual plants each dpi and extracted for antibody quantification using sandwich ELISA. The data are shown as the mean ± standard deviation (SD) of triplicates.
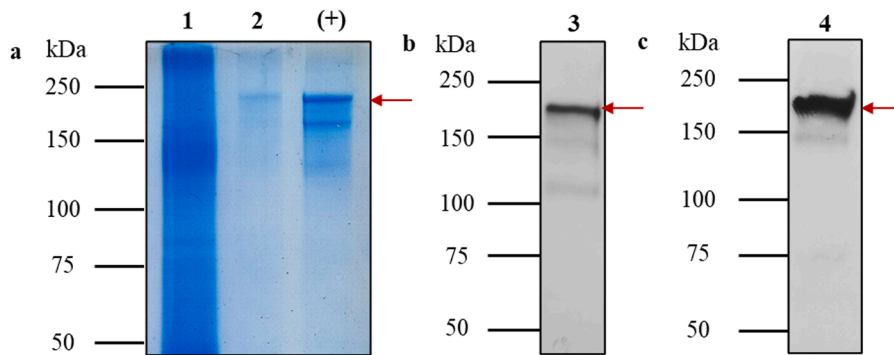

Fig. 3. SDS-PAGE of purified plant-produced anti- Δ9-THC mAb under non-reducing and reducing conditions. After purifying crude extracts, the purified product was analyzed by SDS-PAGE staining with Coomassie blue stain (a, d). For western blot analysis, the purified product was separated in the polyacrylamide gel and transferred to a nitrocellulose membrane, and the membrane was incubated with either HRP-conjugated anti-human gamma chain antibody (b, e) or anti-human kappa chain antibody (c, f) to detect the expected protein band. Lane 1: Crude extracts of infiltrated leaves; Lane 2–4: Purified plant-produced anti-Δ9-THC mAb; Lane (+): Human IgG1 kappa isotype antibody (Positive control).

a

b
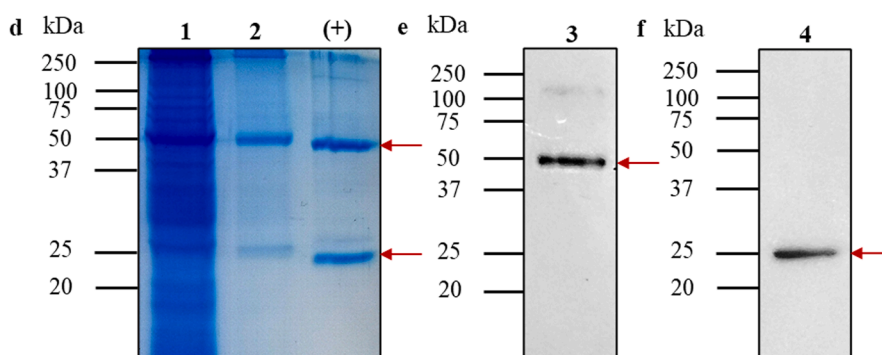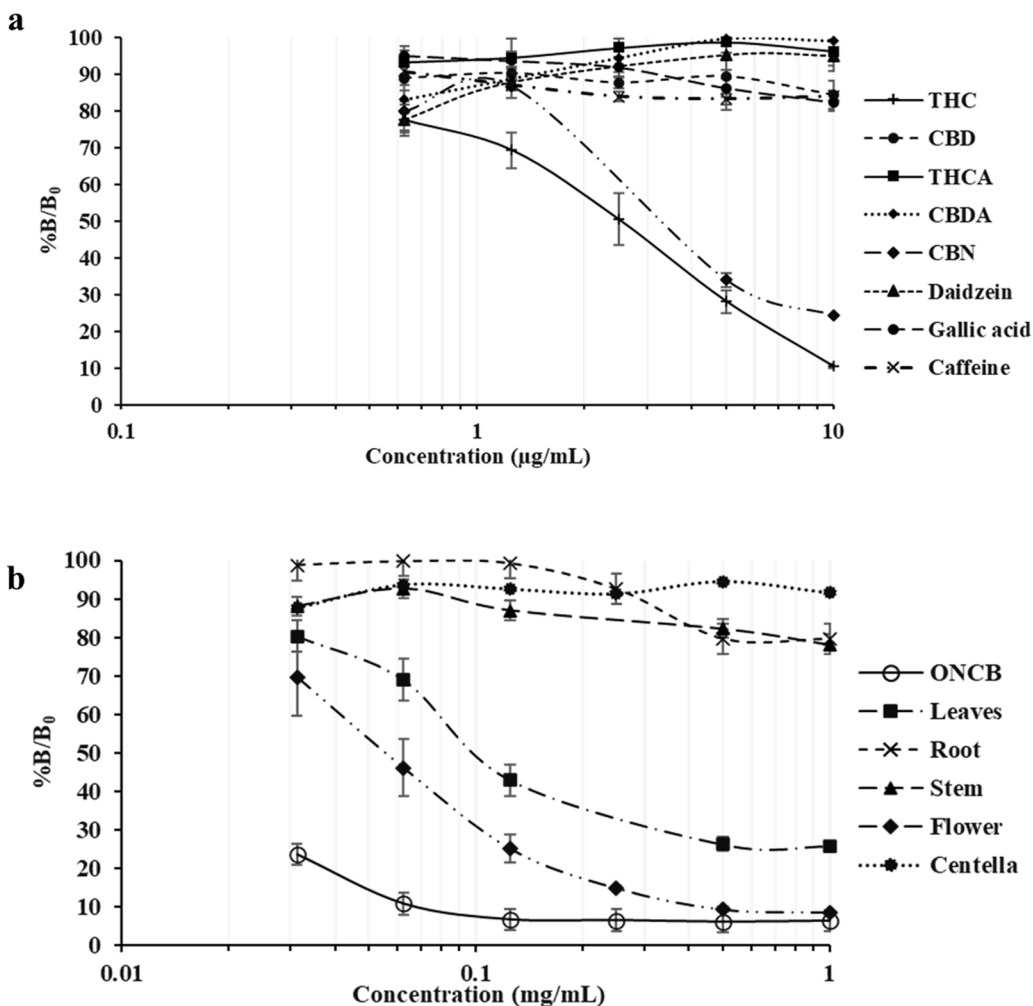
Fig. 4. Binding properties of plant-produced anti- Δ9-THC mAb. Competitive ELISA was performed to investigate the specific binding. The purified plant-produced anti- Δ9-THC mAb were incubated with the reference standards (a) or crude extracts form *Cannabis* plant (b) and added into the 96-well plates coated with THC-BSA conjugated. The bound antibody was detected with HRP-conjugated anti-human kappa antibody. THC, Δ 9-tetrahydrocannabinol; CBD, Cannabidiol; THCA, Δ 9-tetrahydrocannabinolic acid A; CBDA, cannabidiolic acid; CBN, Cannabinol; ONCB; marijuana extracts from office of the narcotics control board.

3.4. Quantification of Δ9-THC by HPLC analysis

The cannabinoid profiles of the hemp and marijuana samples were determined using the HPLC method. In this study, seven major cannabinoids were detected. HPLC chromatograms of the cannabinoids in all samples were obtained (Fig. 5). The cannabinoids in the tested samples were identified by comparing the retention time and UV spectrum with the pure standard. The observed retention times were 5.681, 6.026, 6.365, 6.729, 9.865, 11.972 and 15.028 min for the CBDA, CBGA, CBG, CBD, CBN, Δ9-THC and THCA standards, respectively (Fig. 5a). Four cannabinoids containing CBDA, CBGA, CBD, and THCA were detected in both inflorescences and leaf extracts of hemp (Fig. 5b-c), whereas in hemp stems and roots, only CBDA and CBD were detected (Figs. 5d-e). The acid form of cannabinoids was found in these samples. Because the samples were not heated at the time of extraction, the acid form was not decarboxylated to the neutral form. In addition, Δ9-THC is known to be low in hemp. As a result, the peak of Δ9-THC was not observed in any hemp sample, but Δ9-THCA was still detected. Meanwhile, all major cannabinoids in the marijuana sample were in neutral form: CBG, CBD, CBN, and Δ9-THC (Fig. 5f).

The quantity of Δ9-THC detected in all samples is shown in Table 1. The calibration curve with regression equation $Y = 21.819x + 11.07$ with a correlation coefficient (r^2) of 0.999 for THCA and $Y = 21.273x + 8.26$ with correlation coefficient (r^2) of 0.999 for Δ9-THC were used for quantification. As expected, Δ9-THC was found in higher amounts in marijuana than in hemp. The highest level of Δ9-THC was obtained in the marijuana sample (5.09% w/w of extract). For hemp, although Δ9-

THC is absent, THCA can account for the total amount of Δ9-THC by using the equation Δ9-THC + 0.877*THCA. Table 1 presents the amount of Δ9-THC, THCA, and total number. The total amount of THC quantified in the hemp inflorescences was significantly higher than that in the hemp leaves. The total amount of Δ9-THC was only found in inflorescences, and the leaf extracts contained 1.01 and 0.31% w/w (approximately 0.09 and 0.03% w/w of dried weight), while both THCA and Δ9-THC were absent in hemp stem and root extracts. These results confirm that the Δ9-THC content in our hemp remained lower than the defined legal limit, which should not be higher than 1% w/w of dried plant. In this study, the limit of detection (LOD) and limit of quantification (LOQ) for Δ9-THC using HPLC analysis were 1.48 and 4.48 μg/mL, respectively. The results suggest that the samples with less Δ9-THC than the LOD and LOQ cannot be detected using the HPLC technique. This is a limitation of the technique.

4. Discussion

According to the *cannabis* use detection, conventional analysis was conducted by a complex system with expensive instrument and required specialists. Moreover, it presented some limitation in detection at the low Δ9-THC concentration. Alternatively, immunoassay is a bio-analytical method based on the specific binding between an antibody and the target analytes through ELISA [44, 45]. The immunoassay method plays an important role in various applications such as the diagnosis of diseases, pesticide screening, hapten analyte detection and drug abuse testing [46–48]. This method offers a high-sensitivity,
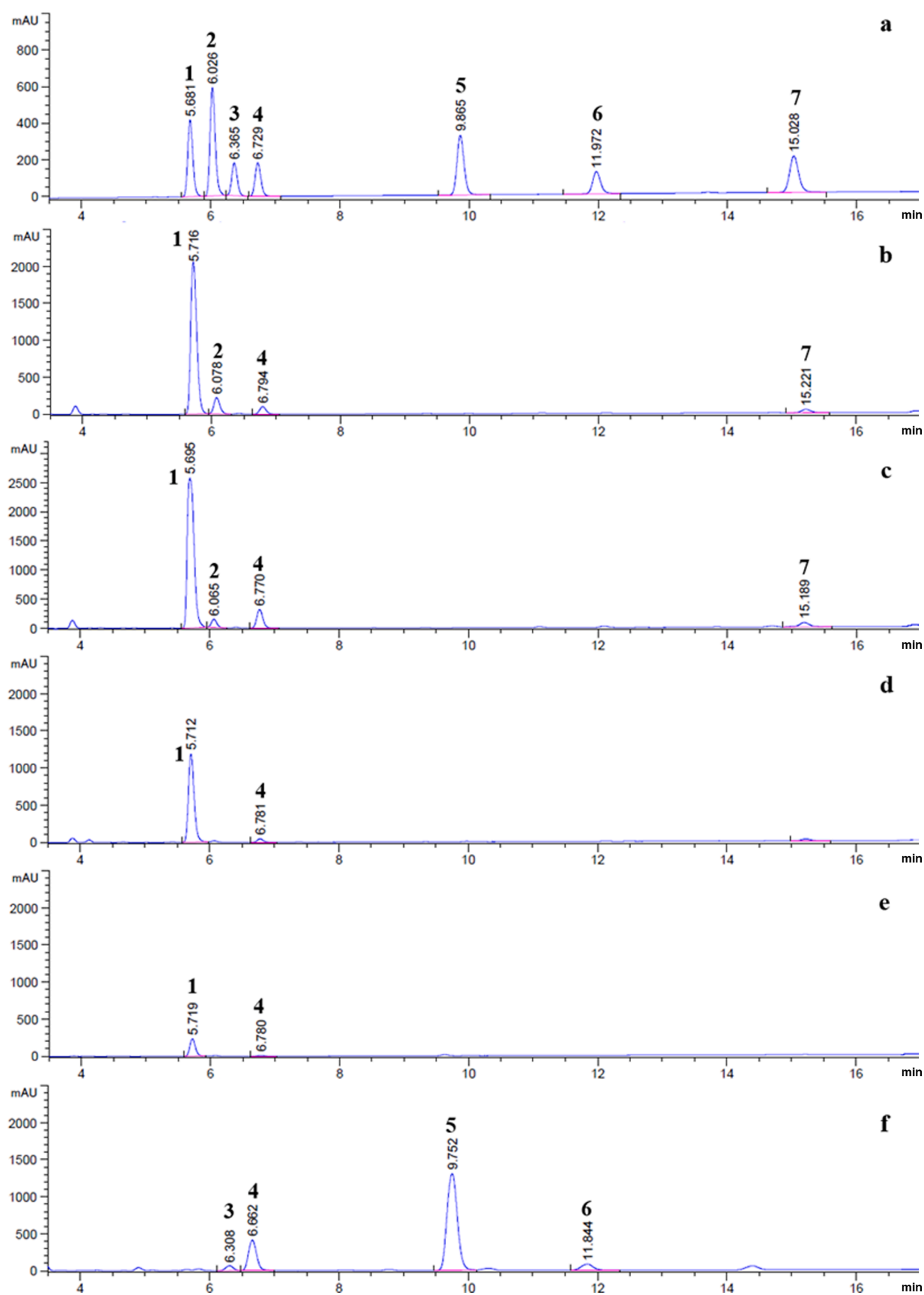
Fig. 5. Chemical profile of cannabinoids in various samples. a; seven cannabinoid standards (1; CBDA 2; CBGA, 3; CBG, 4; CBD, 5; CBN, 6; Δ 9-THC and 7; THCA respectively), b; hemp inflorescences, c; hemp leaf, d; hemp stem, e; hemp root and f; marijuana from ONCB.

Table 1

Δ 9-THC content in hemp and marijuana samples (%w/w of extract). Statistical analysis was performed using GraphPad Prism 9.1, one-way ANOVA, Dunnett's multiple comparisons test, $**p < 0.01$) compared with Hemp inflorescences.

Sample	(%w/w in extract)		
	Δ 9-THC	THCA	Total Δ 9-THC
Hemp inflorescences	ND	1.15	1.15
Hemp leaf	ND	0.35**	0.35
Hemp stem	ND	ND	0.00
Hemp root	ND	ND	0.00
Marijuana	5.09	ND	5.09

Note: Total THC = THC + THCA *0.877.

high-specificity, fast, convenient and cost-effective method of detection. Moreover, it can be simultaneously applied to many samples [49]. According to a previous study, this method was developed to monitor pesticide contamination in the environment [15]. Moreover, we compared the immunoassay and various conventional methods for analyzing mycotoxin contamination in food. The study showed major advantages, including a significant decrease in average analytical time and high specificity to the target [14]. According to Wongta, Hongsibsong [50], the development of an immunoassay can be applied to detect amyloid beta peptides (Aβ1–42), which are related to Alzheimer's disease (AD) and dementia, and it provides an alternative method to diagnose high-risk and early stages of AD with rapid and simple analysis. This method is related to the binding activity between the antibody and the antigen or analyte using enzyme-linked immunosorbent assays (ELISAs) [18, 51]. However, the diagnostic reagents, including antigen, single-chain variable fragment (scFv) or antigen-binding fragment (Fab) of antibody and protein, have been commonly derived from *E. coli* [52–54]. Although the bacterial expression system shows the beneficial aspects of a simple and inexpensive platform, the presence of improper folding and aggregation may affect the binding activity [20, 55]. As shown in a previous study, *E. coli*-derived dengue virus NS1 protein presented improper folding and aggregated protein [56, 57]. Currently, many plant-produced recombinant proteins have been developed for immunoassays. According to He, Lai [58], plant-produced West Nile virus (WNV) antigen and E16 mAb were successfully developed for diagnosis with high specificity and sensitivity. Moreover, the dengue virus NS1 antigen was transiently expressed from *N. benthamiana* and applied to dengue diagnostic testing [59]. During the pandemic situation of coronavirus disease 2019 (COVID-19), receptor binding domain (RBD) and CR3022 mAb were produced in plants with a short production time and could be applied as a diagnostic reagent [34, 60]. For infectious diseases in the swine industry, the plant-produced N-Protein of porcine reproductive and respiratory syndrome virus (PRRSV) can detect antibodies in serum from pig samples. These results suggest that the plant-produced protein can be a diagnostic antigen [61]. Moreover, Rattanapisit, Kitisripanya [62] demonstrated that the plant-produced antibody could protect against small molecules such as miroestrol with high sensitivity and comparable specificity to mAb from hybridoma cells. Previous studies have extended that plant-expression systems are a cost-effective platform to produce recombinant proteins for immunoassay applications.

In a previous study, an anti-Δ9-THC Fab fragment (T3) was produced from an *E. coli* expression system. The anti-Δ9-THC T3 Fab showed binding to Δ9-THC and metabolites of THC, including 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (11-nor-Δ9-THC—COOH) and 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-Δ9-THC) [40]. In our study, *N. benthamiana* was used as an alternative platform to produce an anti-Δ9-THC mAb. The results reveal that the mAb was successfully transiently expressed in *N. benthamiana* by using a geminiviral expression system. This viral expression system, which was modified from the bean yellow dwarf virus genome structure, presented a high protein expression level [41]. Previous studies have shown that geminiviral replicon systems can transiently express many recombinant proteins

such as antigens, antibodies, therapeutic proteins, and growth factors. According to Rattanapisit, Phakham [63], the plant-produced anti-PD1 mAb was transiently expressed with the highest levels at 6 dpi, approximately 0.14 mg/g leaf fresh weight. During the COVID-19 pandemic, the RBD of SARS-CoV-2 and anti-SARS-CoV monoclonal antibodies (mAbs) CR3022, H4 and B38 were rapidly expressed in *N. benthamiana* [34, 64-66]. Moreover, plant-produced ACE2-Fc fusion protein for SARS-CoV-2 treatment was produced at 0.1 mg/g leaf fresh weight after 6 dpi [67]. In addition, plant-produced anti-RANKL mAB with the potential for osteoclast inhibition was rapidly produced after 8 dpi at approximately 0.5 mg/g leaf fresh weight [68].

The results of this study illustrate that the plant-produced anti-Δ9-THC mAb was rapidly expressed with the highest expression level of 0.33 µg/g fresh weight after 4 dpi, and the expression was not observed at 6 and 8 dpi (Fig. 2b). Related to Diamos and Mason [69], a geminiviral expression system from the BeYDV replicon could rapidly express a very high level of recombinant proteins. The high accumulation of protein yield might affect the plant hypersensitive response and activate the pathogen defense mechanism, which caused plant cell death. The study showed that the decrease in Rep and RepA protein expression could reduce the mortality rate of plants and increase the desired protein yield. Here, we did not determine the expression of Rep and RepA proteins, but our results reveal that anti-Δ9-THC mAb can be produced in plants and bind to Δ9-THC. In future studies, other plant expression vectors will be tested to improve the recombinant protein yield.

The specificity and sensitivity of the antibody were determined by competitive ELISA. A previous study presented the binding properties of the anti-Δ9-THC T3 Fab produced from *E. coli*. The results demonstrated the specificity and sensitivity through competitive ELISA, which could be developed for Δ9-THC detection [40]. Our results represent the sensitivity and specificity binding of mAb to Δ9-THC and the metabolites of Δ9-THC such as cannabinol (CBN) among various reference standards (Fig. 4a). The results show cross-reactivity of the plant-produced mAb to CBN, which is the nonenzymatic oxidation by product of THC and most common artifact after prolonged storage. However, the immunoassay with anti-Δ9-THC mAb can be used as the screening method, followed by other highly specific methods such as HPLC and GC.

Moreover, the binding activity of plant-produced mAb was determined with the hemp extracts from different parts, including the roots, stems, leaves, and inflorescences, and marijuana extract. According to the results, plant-produced mAb provided high sensitivity in marijuana from ONCB (positive control), inflorescence and leaf extracts, while the stem, root extract and *Centella* (negative control) were not observed (Fig. 4b). These results suggest that the plant-produced mAb can be applied to screen the fluid samples of marijuana abuse because it can recognize only the cannabis extract. As shown in a previous study, the development of immunocomplexes of the Fab fragment (T3) and THC was tested with other narcotics, including heroin and amphetamine. The results reported no cross reactivity with other drugs [40]. However, our binding result finds a cross reactivity of the mAb to CBN, which is one of the cannabinoids and Δ9-THC-metabolite. Therefore, the binding properties of marijuana may result from the cross reactivity of both Δ9-THC and CBN. More studies should be validated to avoid this cross reaction and provide the clear binding property of the Δ9-THC-metabolite for further cannabis-based food product detection. Our results correlate with a previous report that revealed the content of Δ9-THC in dried seeds, roots, stems, leaves, and inflorescences with n.d., n.d., 0.3%, 0.8%, and 15.2% w/w, respectively [70]. Moreover, the HPLC analysis reveals that the Δ9-THC peak in both inflorescences and leaves of hemp could not be detected. Then, Δ9-THC in both samples was then calculated based on only the THCA content. Interestingly, it was observed that THCA could not bind with the plant-produced mAb. Therefore, a low amount of Δ9-THC in the samples in the results was detected by plant-produced mAb, while it cannot be quantified by HPLC. When

considering the LOQ of HPLC, which was 4.48 ppm (μg/mL), Δ9-THC in the inflorescences and leaves of hemp was below the LOQ. Meanwhile, our developed plant-produced mAb for immunoassay can detect the Δ9-THC content in the range of 0.625–10 ppm (0.625–10 μg/ml). Thus, it is a suitable technique to detect a tiny Δ9-THC quantity to determine whether a suspicious sample complies with the specification of its legal use.

In conclusion, our results demonstrate that anti-Δ9-THC mAbs were rapidly produced in *N. benthamiana* through a geminiviral expression system. The optimal production time was observed on day 4 post-infiltration, and the highest expression level was 0.33 ug/g leaf fresh weight. Furthermore, the binding properties of plant-produced mAb show the specificity and sensitivity binding to Δ9-THC and cannabinol (CBN), which is one of the metabolites of Δ9-THC. Therefore, this proof-of-concept study may promote the concept of a cost-effective and rapid production platform for anti-Δ9-THC mAb and other recombinant proteins for immunoassay applications. However, method validations such as the limit of detection, cross-reactivity and recovery experiments should be evaluated in future studies to develop an accurate immunoassay technique or a lateral flow device for on-site analysis.

Author contributions

W.P. and S.V. designed all the experiments. W.B., K.K. and P.K. performed all of the experiments. All authors analyzed the data and contributed to the manuscript preparation.

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Conflicts of Interests

The authors have no conflicts of interest to declare.

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