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RESEARCH ARTICLE

The Effects of Cannabis Breeding Lines on Nutritional Content and Bioactive Compounds in Cannabis Leaves (Cannabis Sativa L.)

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ABSTRACT

Cannabis is a worldwide economic plant with uses in medicinal, food, nutraceutical, and recreational products. There is a scarcity of data on bioactive compounds and nutritional content to support the proper consumption of cannabis leaves. A completely randomized design was used to determine and quantitatively analyze the content of bioactive compounds and nutritional contents in four cannabis breeding lines at a significance level of $p<0.05$. The four breeding lines have different levels of bioactive compounds and nutritional contents. RSU09 had the highest antioxidant content, followed by RSU12, RSU08, and RSU01. Anthocyanin content was highest in RSU09, followed by RSU08, and lowest in RSU01 and RSU12, respectively. Anthocyanin contents in four cannabis breeding lines were statistically significantly different ($p<0.05$). Each breeding line has different levels of nutritional value. The nutritional contents obtained in this study could provide baseline information on cannabis as a food source and its consumption requirements. Additionally, this study's nutritional contents and bioactive compounds showed potential for broad applications in the food, medical, and nutraceutical industries.

INTRODUCTION

Cannabis (Cannabaceae) or hemp (Cannabis sativa L. subsp. sativa or Cannabis sativa L. subsp. indica) has become a worldwide economic plant that contains multiple bioactive compounds with wide-ranging health benefits for humans. Cannabis contains Δ^9-tetrahydrocannabinol (Δ^9-THC) at more than 0.3%, while hemp, as the fiber type, has Δ^9-THC at less than 0.3%. Bioactive compounds in Cannabis include alkaloids, cannabinoids, fatty acids, terpenoids, flavonoids, phenolics, polysaccharides, and proteins (Liu et al., 2022). Cannabinoids such as cannabidiol (CBD), cannabinol (CBN), Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabigerol acid (CBGA), and Δ^9-tetrahydrocannabinol acid (Δ^9-THCA) are a group of terpene phenolic compounds found in cannabis plants. Δ^9-THC is a psychoactive cannabinoid substance, while the other nonpsychoactive substances (Pellegrini et al., 2005). CBD is an active pharmacological substance used in medicinal applications (Glivar et al., 2020), while CBN

and Δ^9-THCA are minor constituents in fresh cannabis plants (Moreno et al., 2020). These cannabinoids do not occur at significant concentrations in plants. The efficient production of cannabinoids is important for developing dosage to facilitate the successful use of Cannabis (Wang et al., 2016). Cannabinoids have clinical applications due to a wide range of properties such as anti-inflammatory, antimicrobial, sedative, and muscle relaxants for treating anxiety, convulsions, depression, and nausea (Blasco-Benito et al., 2018). Plant phenolic compounds can act directly as antioxidants with inhibitory effects on carcinogenesis and mutagenesis in humans (Saboonchian et al., 2014). Flavonoids comprise the largest group of phenolic compounds, while cannabinoids are the main bioactive compounds found in Cannabis (Farha et al., 2020; Liu et al., 2022). Bioactive compounds are produced in the stems, leaves, and flowers of cannabis plants.

In Thailand, since 2020, Thai legislation has permitted Thais to produce Cannabis for academic research and therapeutic purposes. Other than the flowers, other parts of the cannabis plant may be legally used in cooking (Sommano et al., 2022). However, scant scientific evidence concerning the nutritional contents and bioactive compounds present in Cannabis is available compared to the robust and qualitative evidence supporting the use of Cannabis for medicinal purposes, including the treatment of chemotherapy-induced nausea and vomiting and treatment-resistant epilepsy, neuralgia, and muscle spasms. Cannabis is reported in many Thai traditional medicine textbooks, such as Phra Narai's Elemental Scriptures and a textbook detailing the stone inscriptions in Wat Phra Chetuphon Wimonmangkalaram. Besides the evidence supporting cannabis use for medicinal purposes, Cannabis has also been used for textile purposes (Hakeem et al., 2022; Popijan and Sonsnam, 2016; Sommano et al., 2020). Cannabis fiber is composed mainly of polysaccharides, cellulose, hemicellulose, and pectin, which are readily biodegradable. The stems can make better synthetic paper than perennial plants because Cannabis has a shorter life cycle, a higher yield, and does not require chlorine, unlike papermaking from wood (Liu et al., 2016).

Currently, Cannabis leaves or other plant parts are used in many Thai and foreign dishes. Cannabis leaves are also used in beverages such as tea, coffee, fruit juices, and cannabis-infused sodas (Nizar et al., 2022; Rasera et al., 2021; Saravanan et al., 2022). However, scant data is available in Thailand about the nutritional contents and bioactive compounds of Cannabis to support the proper consumption of cannabis leaves.

This study aimed to investigate the effect of cannabis breeding lines on the nutritional content and bioactive compounds in cannabis leaves (Cannabis sativa L.) to support its proper consumption. More comprehensive data are required to advance the commercialization of Cannabis in the food, medical, nutraceutical, and beauty industries.

MATERIALS AND METHODS

Determination and analysis of moisture content

Five grams of fresh cannabis leaves were weighed, heated, and dried in a hot oven at 105°C. The moisture can was then placed in a desiccator and weighed. This process was repeated until the weight stabilized to within 0.1 g (Nielsen, 2017).

Determination and analysis of ash content

A ceramic crucible was heated at 102±3°C for 3 hr, cooled in a desiccator, and weighed. A 5 g cannabis leaf was burned in an electric furnace at 550°C for 2–3 hr. The ash content was calculated from the equation: Ash content (%) = $\frac{(W_2-W)}{W_1}$ "×100" where W is the crucible weight after drying (g), W_1 is the weight of the pre-dried crucible and sample before burning (g) and, W_2 is the weight of the pre-dried crucible and sample after burning (g) (Harris and Marshall, 2017).

Determination and analysis of fat content

A 2 g cannabis leaf was placed in a Soxhlet distillation apparatus. Petroleum ether (150 ml) was added and heated to 175-325°C for 6-8 hr. The extracted fat was dried at 105°C. The fat content was obtained using the equation: Fat content (%) = $\frac{(w_1-w_2)}{w}$ where W_1 is the weight of the round-bottom flask and the extracted fat (g), W_2 is the weight of the flask (g), and W is the initial sample weight (g) (Nielsen et al., 2017).

Determination and analysis of protein content

Weighing a 1 g sample, 7 g of K_2SO_4 and 0.8 g of Cu_2SO_4 were added. Then, 15 mL of concentrated H_2SO_4 was added and digested until a clear solution was obtained. Distilled water (50 mL) was added

and homogenized with 75 mL of 35% NaOH in 25 mL of 4% boric acid using a DKL heating digester (Velp Scientifica, Italy). This mixture was titrated with a 0.1 NHCl solution until it changed from green to red. The nitrogen and protein contents were calculated using the equations: N content (%) = $\frac{(T-B)\times 14.007\times 100\times N}{W\times 100}$ and protein content = N content (%) 6.25, where T is the volume of 0.1 N HCl solution used for the sample (mL), B is the volume of 0.1 N HCl solution used for the blank (mL), N is the normality of the titrant, and W is the sample weight (g) (Liu et al., 2016).

Determination and analysis of crude fiber content

The crude fiber was determined with the general method (ISO 5498-1981). For 30 minutes, a 2 g dried cannabis leaf was boiled in 0.255 N and 100 mL H_2SO_4. The remaining residue was filtered and washed. After boiling in 100 mL of 1.25% NaOH, the solution was filtered and washed with 10 mL EtOH. A sample was dried at 105°C for 2 hours, then burned at 550°C. The crude fiber content was calculated using the equation: crude fiber content (%) = $\frac{(w_1-w_2)}{w\times 100}$, where W is the sample weight (g), W_1 is the weight of the crucible, and the residue after drying (g), and W_2 is the weight of the crucible after drying and the residue after burning (g) (Śmiechowska and Dmowski, 2006).

Determination and analysis of antioxidant activity

Cannabis leaves were extracted with 1:2 (w/v) EtOH and centrifuged at 8000 rpm for 24 hr. A 0.5 ml aliquot of the extract was mixed with 3 ml of 0.1 mM DPPH solution (2,2-diphenyl-1-picrylhydrazyl) and kept in the dark for 20 min. The absorption rate was measured at 517 nm (Agilent 8453UV) as A_{517}sample. A_{517}blank was performed without the cannabis aliquot. Total antioxidant activity was calculated as EC_{50}.grams of ascorbic acid per liter of cannabis aliquot (g/L) according to the following equation (Zhang et al., 2019).

$$\text{Antioxidant} = \frac{(A_{517}\ \text{blank} - A_{517}\ \text{sample})}{A_{517}\ \text{blank}}$$

Determination and analysis of total phenolic content

Cannabis leaves were extracted with 1:2 (w/v) EtOH and centrifuged at 8000 rpm for 24 hr. A 0.1 mL of supernatant was added to 2 mL distilled water and 0.2 mL of Folin-Ciocalteu phenol reagent. A 1 mL aliquot of 20% Na_2CO_3 was added and kept in the dark. Total phenolic content corresponded to the absorbance of standard gallic acid at 765 nm and was expressed as mg of Gallic Acid Equivalent (GAE) per liter of cannabis solution (mg GAE/L) (Fig. 1) (Khan et al., 2018).

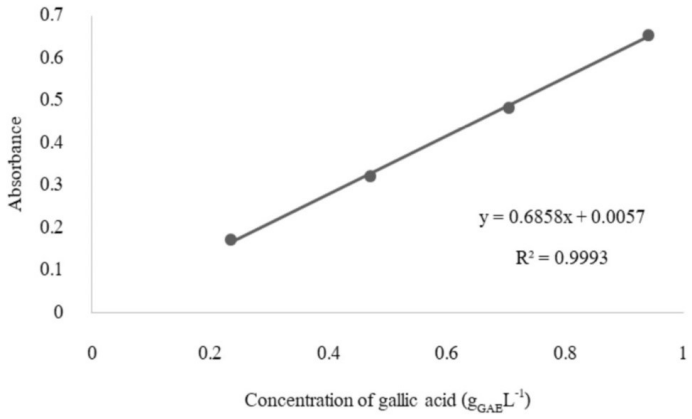

Figure 1: Graph of absorbance at 765 nm against gallic acid concentration

Determination and analysis of anthocyanin content

A fresh cannabis leaf weighing 1 g was weighed and ground. Then, 1% HCl was added to 10 mL of methanol and kept at room temperature for 24 hr before centrifuging at 8000 rpm for 5 min. The clear supernatant was kept under dark conditions. The absorbance was measured at 530 nm, and the total anthocyanin content was calculated using the following equation:

Anthocyanin $\left(\mathrm{mg_{GAE}} \cdot 100\,\mathrm{g_{FW}}^{-1}\right) = [(27.208 \times \mathrm{A_{530}} + 0.0591) \times$ solution volume $(\mathrm{mL})] \times \frac{10}{1000} \times$ solution volume (g) (Zhang et al., 2019).

Determination and analysis of chlorophyll content

A 5 mL aliquot of DimethylFormamide (DMF) solution was added to fresh cannabis leaves and stored in a dark place for 24 hr. One milliliter of the extract was measured at 647 and 664 nm, respectively. Absorbance values were recorded, and the chlorophyll content wascalculated by the following equations:

ChlA $= \frac{(-2.99\ \mathrm{A_{647}} + 12.64\ \mathrm{A_{667}}) \times \mathrm{Vol}}{\mathrm{x} \times \text{ area}}$m, Chl B $= \frac{(23.26\ \mathrm{A_{647}} - 5.60\ \mathrm{A_{667}}) \times \mathrm{Vol}}{\mathrm{x} \times \text{ area}}$, and Chl total $= \frac{(20.27 A_{647} + 7.04\ \mathrm{A_{667}}) \times \mathrm{Vol}}{\mathrm{x} \times \text{ area}}$ (Aminot and Rey, 2014).

Determination and analysis of cannabigerolic acid, cannabidiol, Δ^9-tetrahydrocannabinol and Δ^9-tetrahydrocannabinolic acid contents

All chromatographic runs were carried out using a Hewlett-Packard (HP) High-Performance Liquid Chromatography (HPLC) system, consisting of a G1311A quaternary solvent pump (1200 series), a G1322A solvent degasser (1200 series), a G1313A auto sampler (1100 series) and a G1316A column compartment (1100 series). A Waters 2996 Photodiode-Array Detector (PAD) was used for detection. Full spectra were recorded in the range of 200-400 nm. Chromatographic separations were achieved using a Waters XTerra® MS C18 analytical column (5 µm, 250 mm × 2.1 mm id.), protected by a Waters XTerra® MS C18 guard column (5 µm, 10 mm × 2.1 mm id.). Equipment control, data acquisition, and integration were performed using Empower Pro 2.0 software.

The mobile phase consisted of MeOH/water containing 50 mM of ammonium formate (pH 5.19). The initial setting was 68% methanol (v/v). The flow rate was 0.3 mL/min, and the injection volume was 30 µL. All experiments were conducted at 30°C (Wang et al., 2018).

Statistical analysis

All analyses were performed in triplicate, and the results were reported as mean data Standard Deviation (SD). The SPSS program (SPSS Inc., Chicago, USA) version 20.0 was used to analyze all data using completely randomized design statistical methods at a significance level of p<0.05.

Ethics statements

No studies involving human participants or animals were performed by any authors.

RESULTS AND DISCUSSION

Bioactive compounds

Bioactive compounds detected in the leaves of four cannabis breeding lines showed statistically significantly different antioxidant contents (p<0.05). RSU09 had the highest antioxidant content, followed by RSU12 and RSU08, and RSU01 had the lowest at 24.61 ± 1.00, 21.26 ± 0.74, 18.07 ± 0.91 and 9.48 ± 0.52 $EC_{50}.gL^{-1}$, respectively. Total phenolic compounds in cannabis leaves did not differ statistically, averaging 2.45 ± 2.50 $mg_{GAE}.100_{gFW}{}^{-1}$. Anthocyanin contents in the cannabis leaves were statistically significantly different (p<0.05). Anthocyanin content was highest in RSU09 followed by RSU08 and lowest in RSU01 and RSU12 at 13.02 ± 0.23, 7.27 ± 0.51, 7.04 ± 0.82 and 6.96 ± 0.44 $mg_{GAE}.100_{gFW}{}^{-1}$, respectively. Chlorophyll A content in cannabis leaves differed significantly, with the highest content in RSU12 and RSU08, which were not significantly different from RSU09, and lowest in RSU01, at 49.22 ± 11.43, 43.68 ± 9.43, 40.50 ± 10.21 and 34.34 ± 9.32 mg/cm2, respectively. Chlorophyll B in cannabis leaves was not significantly different, averaging 12.088 ± 10.96 mg/cm2. Total chlorophyll content in cannabis leaves was significantly different and highest in RSU12, with no significant difference between RSU08 and RSU09, and lowest in RSU01 at 61.79 ± 1.56, 56.27 ± 4.54, 52.23 ± 3.55, and 45.80 ± 5.32 mg/cm2 respectively, as shown in Table 1. Antioxidants and free radicals were used in disease mechanisms such as cancer and atherosclerosis, two significant causes of death. Lobo et al. (2010) reported that atherosclerosis due to free radical reaction involving diet-derived lipids induces cell injury and changes the arterial walls. Meanwhile, phenolic compounds in plants can act directly as antioxidants, inhibiting carcinogenesis and mutagenesis in humans

(Saboonchian et al., 2014). Anthocyanins (ACNs) are water-soluble phenolic compounds of natural pigments such as violet, red, orange, and blue colors (Riaz et al., 2016). ACNs have many pharmacological effects in preventing cancers, cardiovascular disease, antitumoral agents, anti-obesity, anti-diabetic effects, and anti-Alzheimer (Rasera et al., 2021; Parveen et al., 2019; Xie et al., 2018). These findings indicate that the bioactive compounds in cannabis leaves are needed to further develop cannabis as a commercial product in the food, medical, nutraceutical, and beauty industries.

Table 1: Bioactive compounds in four cannabis breeding lines

Cannabis Line	Antioxidant ($EC_{50}.gL^{-1}$)	Total Phenolic Compounds ($mg_{GAE}.100g_{FW}{}^{-1}$)	Anthocyanin ($mg_{GAE}.100g_{FW}{}^{-1}$)	Chlorophyll A (mg/cm^2)	Chlorophyll B (mg/cm^2)	Total Chlorophyll (mg/cm^2)
RSU01	9.48 ± 0.52^{d}	2.57 ± 0.32	7.04 ± 0.82^{c}	34.34 ± 9.32^{b}	11.47 ± 10.01	45.80 ± 5.32^{c}
RSU08	18.07 ± 0.91^{c}	2.41 ± 0.81	7.27 ± 0.51^{b}	43.68 ± 9.43^{a}	12.58 ± 4.50	56.27 ± 4.54a^{b}
RSU09	24.61 ± 1.00^{a}	2.42 ± 0.53	13.02 ± 0.23^{a}	40.50 ± 10.21^{ab}	11.73 ± 3.55	52.23 ± 3.55^{bc}
RSU12	21.26 ± 0.74^{b}	2.38 ± 0.42	6.96 ± 0.44^{c}	49.22 ± 11.43^{a}	12.57 ± 2.12	61.79 ± 1.56^{a}
Mean ± SE*	18.35 ± 2.47	2.45 ± 2.50	8.57 ± 0.81	41.49 ± 3.33	12.08 ± 3.31	54.02 ± 2.95

Nutritional contents

The moisture content of four cannabis breeding lines' leaves was not statistically different, with an average of 69.62 ± 2.22%. Ash content was significantly different, with the highest in RSU12 followed by RSU09 and the lowest in RSU08, and RSU01 at 9.39 ± 0.11, 7.30 ± 0.21, 5.63 ± 0.43 and 5.37 ± 0.51 g/100 gFW, respectively. Fat content in the cannabis leaves was significantly different, with the highest in RSU09, RSU08 and RSU12 and the lowest in RSU01 at 11.40 ± 0.24, 11.14 ± 0.31 11.14 ± 0.31, and 7.30 ± 0.22 g/100 g_{FW}, respectively. Nitrogen and protein contents were not significantly different, averaging 1.67 ± 3.94 g/100g_{FW} and 10.44 ± 3.94 g/100g_{FW}, respectively. Crude fiber content was statistically significantly different, with the highest coarse fiber content in RSU12, followed by RSU01 with no significant difference with RSU09, and the lowest in RSU08 at 6.14 ± 0.65, 4.51 ± 0.32, 3.71 ± 0.45 and 2.45 ± 0.37 g/100 g_{FW}, respectively. The carbohydrate content of cannabis leaves did not differ significantly, averaging 5.44 ± 4.52 g/100 g_{FW}. The calorie content of cannabis leaves was significantly different, with the highest in RSU08, RSU09, and RSU12 and the lowest in RSU01 at 163.21 ±0.34, 161.73 ± 0.32, 160.49 ± 0.45, and 122.86 ± 0.54 kcal/100 g_{FW}, respectively (Table 2). These results corresponded with the finding of Audu et al. (2014). Ames et al. (1990) reported that the different varieties of cannabis have different levels of medicinal and nutritional value. The nutritional contents obtained in this study could provide baseline information on cannabis as a food source's consumption requirements.

Table 2: Nutritional contents of four cannabis breeding lines

Cannabis Line	Moisture (%)	Ash (g/100 g_{FW})	Fat (g/100 g_{FW})	Nitrogen (g/100 g_{FW})	Protein (g/100 g_{FW})	Crude fiber (g/100 g_{FW})	Carbohydrate (g/100 g_{FW})	Calories (kcal/100 g_{FW})
RSU01	73.04±0.54	5.37 ± 0.51^{c}	7.30 ± 0.22^{b}	1.46±0.11	9.12±0.32	4.51 ± 0.32^{b}	5.17 ± 0.27^{bc}	122.86 ± 0.54^{b}
RSU08	70.59±0.22	5.63 ± 0.43^{c}	11.14 ± 0.31^{a}	1.63±0.16	10.19±0.21	2.45 ± 0.37^{cd}	8.33 ± 0.52^{a}	163.21 ± 0.34^{a}
RSU09	67.50±0.43	7.30 ± 0.21^{b}	11.40 ± 0.24^{a}	1.83±0.10	11.46±0.15	3.71 ± 0.45^{bc}	3.32 ± 0.77^{c}	161.73 ± 0.32^{a}
RSU12	67.36±0.35	9.39 ± 0.11^{a}	11.14 ± 0.31^{a}	1.76±0.45	10.99±0.22	6.14 ± 0.65^{a}	6.13 ± 0.81^{ab}	160.49 ± 0.45^{a}
Mean±SE*	69.62±2.22	6.92±3.28	10.24±3.83	1.67±3.94	10.44±3.93	4.20±4.23	5.44±4.52	152.07±3.35

*Mean values with different superscript letters within each column denote significant ($p < 0.05$) difference between groups; g/100 g_{FW} = gram per 100 grams fresh weight.

Cannabinoid content

The chromatographic cannabinoid fingerprint is shown in Fig. 2. Cannabinoid contents of the four breeding lines of cannabis leaves are shown in Table 3. The Δ^9-THC content of cannabis leaves was not statistically different, averaging 0.10% w/w. The Δ^9-THCA content in cannabis leaves was significantly different, with the highest contents in RSU01 and RSU08, followed by RSU09 and RSU12 at 1.18 ± 0.55, 1.09 ± 0.47, 1.02 ± 0.67, and 1.02 ± 0.65% w/w, respectively. CBD was not found in the four breeding lines of cannabis leaves. Wang et al. (2018) reported that CBD in cannabis decreased with cyclization from CBD to Δ^9-THC, followed by degradation of Δ^9-THCA to CBN when exposed to light. Cannabinoid contents in cannabis are highly variable in terms of genetics and botany. Even though Δ^9-THC and Δ^9-THCA are minor constituents in cannabis leaves, dosage development is important to facilitate the success of cannabis use (Wang et al., 2016).

Table 3: Cannabinoid content of four cannabis breeding lines

Cannabis line	Δ^9-THC (% w/w)	Δ^9-THCA (% w/w)	CBD (% w/w)
RSU01	0.12 ± 0.41	1.18 ± 0.55^{a}	0
RSU08	0.09 ± 0.33	1.09 ± 0.47^{ab}	0
RSU09	0.10 ± 0.26	1.02 ± 0.67^{b}	0
RSU12	0.08 ± 0.35	1.02 ± 0.65^{b}	0
Mean ± SE*	0.10 ± 4.29	1.08 ± 2.03	nd

*Mean values with different superscript letters within each column denote significant (p < 0.05) difference between groups; Δ^9-THC = Δ^9-tetrahydrocannabinol; Δ^9-THCA = Δ^9-tetrahydrocannabinolic acid; CBD = cannabidiol.

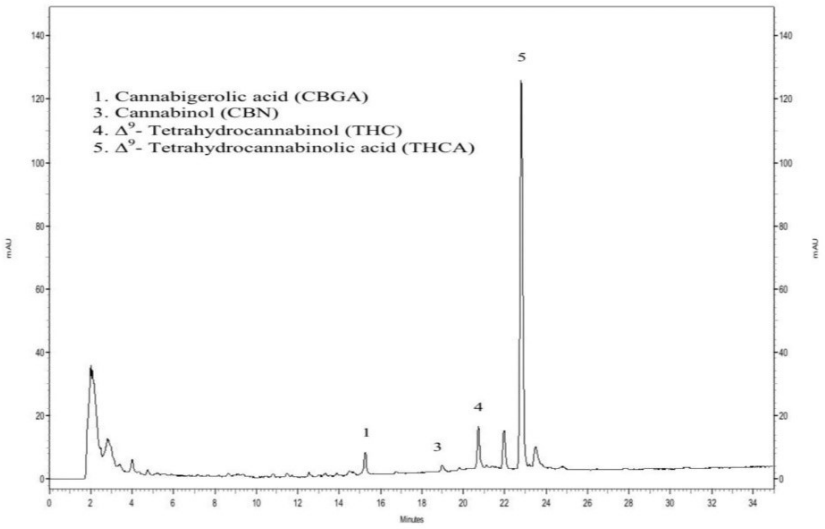

Figure 2: Chromatographic fingerprint (HPLC) of cannabis leaves

CONCLUSION

This study represents a comprehensive nutritional content and bioactive compounds in cannabis leaves (Cannabis sativa L.). Results indicated that the four breeding lines showed slightly significant differences in the nutritional content and bioactive compounds of cannabis leaves. The cannabis breeding line RSU09 had the highest nutritional content and bioactive compounds, but results were not significantly different from RSU12, followed by RSU08 and RSU01, which had the lowest nutritional content. Δ^9-THC and Δ^9-THCA were found in fresh cannabis leaves. The active ingredients such as antioxidants, anthocyanin, total phenolic compounds, protein, crude fiber, fat, and carbohydrate found in these cannabis breeding lines have varying potencies and modes of action depending on whether applied for broad applications in the food, medical, nutraceutical and beauty product industries. In the food industry, cannabis plant parts

have been used as ingredients, such as hemp coconut oil, hemp butter, or hemp mayonnaise. In Thai cuisine, young leaves are eaten as a vegetable with chili paste, while mature leaves are added to dishes such as tom yum, noodles, and stir-fries or to other national dishes such as pot brownies and cannabis cookies. However, theoretically, the daily nutritional needs of adults are 2000 kcal (men) and 1600 kcal (women); therefore, daily consumption of 1.2 kg and 989 g of cannabis leaves would be required, respectively, for humans to achieve their daily sustenance from cannabis leaves. These nutritional content and bioactive compounds findings obtained in this research can form baseline information for the possible utilization of these cannabis leaves for subsequent utilization in supplementing food, medical, nutraceutical, and beauty products, as well as its overall importance in sustaining biodiversity.

Conflicts of interest

None declared.

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