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Report

Chemical composition of seized cannabis and its extraction for medical purposes

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Abstract: *Cannabis indica* obtained from the Office of the Narcotics Control Board (ONCB) of Thailand was examined for medical purposes. The ONCB cannabis was seized and distributed to our research group for investigation of chemical compositions and purification of major cannabinoids. Its essential oil was analysed and aromadendrene was identified as the major terpene. Small amounts of cannabinoids were also observed. The components of the seized cannabis were extracted using ethanolic extraction and supercritical CO_2 extraction. All crude extracts were analysed using high-performance liquid chromatography coupled with a cannabinoid analyser. All extracts contained cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabinol (Δ^9-THC) as the major constituents. A preliminary study showed notable anticancer activities of CBD, CBN and Δ^9-THC against cervix carcinoma cell and epithelial human breast cancer cell.

Keywords: cannabidiol, cannabinol, Δ^9-tetrahydrocannabinol, seized cannabis, cannabinoids, cannabis extraction, anticancer activity

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INTRODUCTION

Cannabis indica is a plant originating from central Asia and is commonly called cannabis, marijuana, ganja or Indian hemp [1-4]. It is illegal in most countries due to its high levels of Δ^9-tetrahydrocannabinol (Δ^9-THC), a psychoactive compound with allied physiological effects**.** In the US, cannabis was classified as a substance which has no approved medical use and has a high potential for abuse [5]. This federal definition is highly controversial and can limit the availability of cannabis for clinical research. However, many US states have legalised the use of cannabis for medical and/or recreational use [6-8]. Prescription medicines containing synthetic cannabinoids (THC) are approved by the Food and Drug Administration to treat certain conditions [9]. Another important substance is cannabidiol (CBD), which is non-psychoactive and non-addictive and has high bioavailability. CBD has well-documented anti-inflammatory properties and is used to treat insomnia, chronic pain, arthritis and joint pain [10-16]. This compound is incorporated into a variety of products from drinks, pet products, lotions to chewing gums [17-20].

In Thailand people who used to possess and deal with cannabis could incur harsh penalties. The Office of the Narcotics Control Board (ONCB) reported the seizure of 14342.71 kg of illegal marijuana plants in 2019 [21] and most of these were destroyed by incineration, in accordance with the ONCB's regulations. Of this amount, ONCB distributed 1,739 kg to medical and research institutions authorised to use the plant for research purposes. This distributed cannabis has been tested for cadmium toxicity and pesticide residue to ensure its safety for research and consumption. Research agencies have tried to develop it into medical cannabis for application in modern and alternative medicine. At least 800,000 bottles of medical cannabis oil or CBD are produced [21] to treat several diseases including Parkinson's and Crohn's disease. The essential oil (EO) in cannabis contains a mixture of volatile compounds, mainly monoterpenes, sesquiterpenes and other terpenoid-like substances [23-26], with antidepressant, anxiolytic, sedative, antimicrobial, and antioxidant properties [26-31]. Apart from THC and CBD, many other related structures in cannabinoids may influence pharmacological properties. However, the storage conditions of cannabis material, e.g. temperature, light and duration of storage, may affect the content of important ingredients. For example, THC is degraded at high temperatures [32-34].

With cannabis now legalised, we are interested in the content of its major ingredients. The seized cannabis which has been stored for one and two years was extracted by three different methods and its chemical components were studied in comparison with previous reports. The purified cannabinoids were also preliminarily studied for their anticancer properties.

MATERIALS AND METHODS

Cannabis Samples

Two samples of dried cannabis were obtained from the ONCB: one (S18Th) was obtained in February 2017–February 2018; another one (S19Bd) was seized in 2019 while it was smuggled from a neighboring country.

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Cannabis Extraction

Steam distillation

A dry sample of S18Th (1 kg) was steam distilled for 2 hr using a Clevenger-type apparatus and a pale yellow EO was obtained. The oil was analysed by gas chromatography–mass spectrometry (GC-MS). The plant residue (S18Rs) was used for further ethanol extraction.

Ethanol extraction

Dried cannabis (1 kg each of S18Th, S19Bd and S18Rs) was soaked in 95% ethanol for 5 days. Then the solvent was removed from the resulting extract solution by a rotary vacuum evaporator to obtain the crude extract.

Supercritical CO_2 extraction

A total of 1 kg of S18Th was extracted by a supercritical CO_2 extractor (Model HA120-50-05-C, Nantong Huaan Supercritical Extraction Co. China). Cannabis was placed in an extraction vessel and pressurised with CO_2. The liquid CO_2 was brought to a supercritical state before entering the extractor vessel. The extracting conditions were set up for the compression system at 37°C with a pressure of 4.5 MPa for 120 min. to obtain the crude cannabis extract.

Identification of Cannabinoids

The EO and crude cannabis extracts were analysed by GC-MS (Shimadzu, model GC-MS-QP2010 Ultra). Helium was used as the carrier gas (1.17 mL/min.) in a capillary column (SLB-5 MS; 30.0 m x 0.25 mm (id); 0.5 μm) with injector temperature of 280°C, detector temperature of 220°C/MS (EI), and oven temperature programmed at 5 min. isothermal at 60°C and then 10°C/min. to 230°C. The injection volume was 1.0 mL. Relative percentage amounts were calculated from the peak total area by an apparatus software. The compounds were identified by comparing their retention times and mass spectra with those obtained from the MS library.

Cannabinoid concentrations were quantified on a Shimadza Prominence high-performance liquid chromatography (HPLC) system connected with a cannabinoid analyser. Separation was carried out using 0.085% (w/w) phosphoric acid in water : 0.085% (w/w) phosphoric acid in acetonitrile (85 : 15) as mobile phase, a flow rate of 1. 6000 mL/ min., injection amount of 15 μL, and analysis time of 15 s at 35°C. The detector model was LC-2030/ 2040 PDA 220, 254 nm and the column was Shim-pack GISS column HPLC Agilent Model LC-2030 Controller, size: 5 μm, C18, 4.6 x 150 mm (id). Standard substances used were cannabidivarin (CBDV), CBD, cannabinol (CBN), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), tetrahydrocannabivarin (THCV), Δ^8-tetrahydrocannabinol (Δ^8-THC), Δ^9-THC, cannabichromene (CBC) and tetrahydrocannabinolic acid (THCA).

Purification of THC, CBD and Related Cannabinoids

The separation of THC, CBD and related substances was carried out by column chromatography of S18Th (CO_2 extraction) using a silica gel 60 from Merck (0.063–0.200 mm) as an adsorbent and eluting with hexane: EtOAc (10:1). The structures of the purified active substances were confirmed by nuclear magnetic resonance (NMR) spectra obtained from Bruker AVANCE 300 with $CDCl_3$ as solvent and tetramethylsilane as internal standard. The

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contaminating heavy metals were analysed using inductively coupled plasma MS (ICP-MS) detector Agilent 7900.

Cytotoxicity

The inhibition of the growth of cancer cells (HeLa and MDA-MB-231, Korean Cell Line Bank, South Korea) and cytotoxicity (LLC-MK 2 and Vero cell lines, Korean Cell Line Bank, South Korea) were tested in the crude extracts by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The extractable substances were CBG, CBN, CBD and a mixture between Δ^8-THC and Δ^9-THC. Cell cultures in Dulbecco's modified eagle medium containining 10% fetal bovine serum at 37°C and 5% CO_2 was prepared to achieve a density of $1x10^5$ cells/mL in a 96-well microplate at each concentration. The cultures were incubated at 37°C and 5% CO_2 for 24 hr, food was replaced with a new one, and the cultures were added with 400 µg/mL MTT solution to the volume of 100 µL and incubated at 37°C and 5% CO_2 for 4 hr. The food was removed and dimethyl sulfoxide was added at 100 µL/well. Formazan colouration was measured at the absorbance of 540 nm with an enzyme-linked immunosorbent assay reader. The experiment was repeated twice; then the IC_{50} and % survival were calculated.

RESULTS AND DISCUSSION

The yield of cannabis EO obtained from S18Th was 0.8 ml (0.085%). The yields of the crude extracts obtained from different samples of cannabis by different methods are presented in Table 1. Analysis of the chemical ingredients of the EO was performed by GC/ MS and the results are shown in Table 2. The terpenes found at the highest amounts are aromadendrene (11.24%), caryophyllene oxide (6.48%), (-)-globulol (5.51%), azulene (5.21%), dl-isopulegol (3.38%), caryophyllene (1.11%), and linalool (0.98%). The main terpene constituents found are markedly different from those previously reported [24], which may be due to the difference in varieties, cultivation conditions and storage conditions. The CBN level is slightly lower than that of CBD while CBG is not detected. Δ^8-THC and CBC are present in small amounts.

Table 1. Yields of crude cannabis extracts

Cannabis sample	Method	Extraction time (hr)	Yield
S18Th	Ethanol extraction	72	17.90 %
S18Bd	Ethanol extraction	72	27.43 %
S18Th	CO_2 Extraction	2	5.9 %
S18Rs	Ethanol extraction	5	21.90 %

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Table 2. Chemical composition of cannabis EO by GC-MS

No.	Compound	RA[a] %	MW[b]	Quality[c] %	Identification[d]
1	aromadendrene	11.24	204	99	1,2
2	caryophyllene oxide	6.48	220	99	1,2
3	(-)-globulol	5.51	222	99	1,2
4	azulene	5.21	128	99	1,2
5	dl-isopulegol	3.38	154	99	1,2
6	linalool	0.98	154	99	1,2
7	caryophyllene[1]	1.18	204	99	1,2
8	Δ^8-THC	0.21	314	99	1,2
9	CBC	0.04	314	99	1,2
10	CBD	0.37	314	99	1,2
11	CBN	0.32	310	99	1,2

[a] Relative area (peak area relative to total peak area)
[b] Molecular weight from GC-MS (EI) data
[c] MS quality compared with database
[d] 1: based on comparison of mass spectra from NIST library; 2: based on comparison of mass spectra from Wiley library

Table 3 shows the analysis results for the crude extracts by GC-MS. The crude extracts of S18Th and S19Bd contain the same type but different contents of cannabinoids. All extracts show the presence of non-psychotropic substances CBD, CBN and CBG; CBN is the main substance and found in the highest amount in the ethanolic extract of S19Bd (49.13% relative area) . The psychotropic substance Δ^9-THC is present in small amounts in both the S18Th ethanolic and supercritical CO_2 extracts. The low amounts of THC might be due to its degradation to CBN by heat or oxygen [36].

Table 4 shows the analysis results for the EO and crude extracts by HPLC. In the EO the major cannabinoid found is CBD (42.88%). CBN and Δ^9-THC are about half whereas CBDV is 1/6 the amount of CBD. All crude extracts contain CBN, Δ^9-THC and CBD except S18Rs. Although GC-MS analysis shows the presence of CBD in S18Rs, its amount might be too low to be detected by HPLC.

The ratio of the active CBD to THC (Figure 1) can be used to determine the effect of a cannabis extract as non-psychotropic or psychotropic. In the case of S18Rs, the CBD/THC ratio cannot be determined since no CBD was detected by HPLC, but a CBD/THC ratio of less than 1 is observed in other extracts except the EO and S19Bd extract. However, this ratio depends on the variety or source of the plant material; the seized cannabis came from different sources including Thailand, which are low in CBD. Heat and storage conditions also affect the stability of the active ingredients in cannabis. At high temperatures, THC can be converted to CBN, thus lowering the amount of THC, and storage conditions can also result in the loss of Δ^9-THC [37].

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Table 3. Cannabinoids detected in the crude extracts by GC-MS

Peak No.	Compound	RA[a]				Quality[b] %	Method[c]
		S18Rs	S19Bd	S18Th	S18Th (CO_2)		
1.	CBDV	nd	0.25	nd	nd	99	1,2
2.	CBDA	nd	nd	nd	nd	99	1,2
3.	CBGA	nd	nd	nd	nd	99	1,2
4.	CBG	0.72	2.48	2.62	2.42	99	1,2
5.	CBD	2.13	25.89	16.84	12.99	99	1,2
6.	THCV	nd	nd	nd	nd	99	1,2
7.	CBN	7.23	49.13	27.73	13.27	99	1,2
8.	Δ^9-THC	nd	nd	0.37	0.59	99	1,2
9.	Δ^8-THC	nd	nd	nd	nd	99	1,2
10.	CBC	nd	nd	nd	nd	99	1,2
11.	THCA	nd	nd	nd	nd	99	1,2

[a] Relative area (peak area relative to total peak area)

[b] MS quality compared with database

[c] 1: based on comparison of mass spectra from NIST library; 2: based on comparison of mass spectra from Wiley library

nd = not detectable

Table 4. Cannabinoids detected in EO and crude extracts by HPLC

Compound	RT*	Relative area (%)				
		EO	S18Rs	S19Bd	S18Th	S18Th (CO_2)
CBDV	2.570	7.32	nd	nd	nd	n/a
CBDA	3.389	nd	nd	1.98	1.43	0.42
CBGA	3.688	nd	nd	nd	nd	nd
CBG	3.854	nd	nd	2.04	2.37	2.49
CBD	4.001	42.88	nd	23.12	15.29	12.23
THCV	4.154	nd	nd	nd	nd	0.67
CBN	5.682	27.19	59.21	63.84	39.93	25.93
Δ^9-THC	6.573	22.58	40.70	1.89	39.18	56.26
Δ^8-THC	6.760	nd	nd	nd	nd	nd
CBC	7.439	nd	nd	2.10	1.78	0.19
THCA	7.672	nd	nd	nd	nd	nd

* Retention time; nd = not detectable

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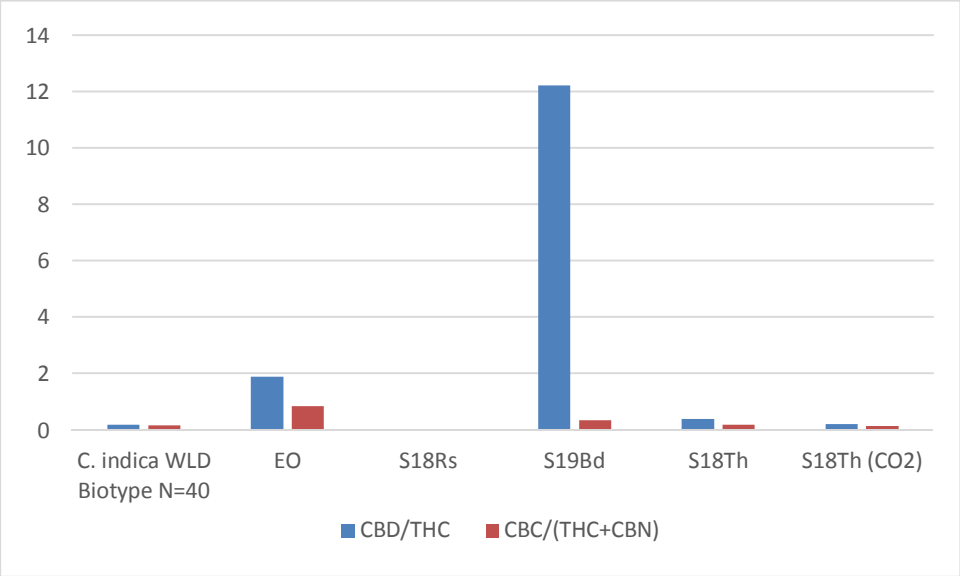

Figure 1. Ratio of CBD/Δ^9-THC and CBD/(Δ^9-THC + CBN) in EO and extracts

A comparison of CBD/(THC+CBN) (Figure 1) is also determined as it is assumed that some THC is converted to CBN. The ratios obtained from the extracts show a slight difference from a selected previous report (Table 5) [1]. Although these ratios were obtained from different sources, THC was found in higher level than CBD in *C. indica*. Thus, the seized cannabis can be used for medical purposes as it contains crucial ingredients of cannabinoids.

Table 5. Ratios of CBD/Δ^9-THC and CBD/(Δ^9-THC +CBN) in EO and extracts

Name	Relative areas (%)					
	C. indica WLD Biotype [1] N=40	EO	S18Rs	S19Bd	S18Th	S18Th (CO_2)
CBD	1.21	42.88	nd	23.12	15.29	12.23
Δ^9-THC	6.49	22.58	40.70	1.89	39.18	56.26
CBN	0.19	27.19	59.21	63.84	39.93	25.93
CBD/THC	0.19	1.90	nd	12.23	0.39	0.22
CBC/(THC+CBN)	0.18	0.86	nd	0.35	0.19	0.15

Note : nd = not detectable

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The seized cannabis might accumulate heavy metals such as lead, cadmium, arsenic, mercury and chromium, which originated from the use of fertilisers and contaminated soils. To be used in food or as medicine the cannabis must therefore be tested for heavy-metal content. The results of the test (Table 6) show, however, that all samples of the crude cannabis extracts, have low levels of heavy metals (Pb, Hg, As and Cd).

Table 6. Heavy metal content in seized cannabis and cannabis extracts

Sample	Metal (mg/kg)			
	As	Cd	Pb	Hg
S18Th	0.086	0.12	ND[1]	ND
S18Th (CO_2)	0.007	0.086	ND	ND
S19Bd	0.31	0.48	ND	ND
S18Rs (boiled)	0.091	0.17	ND	ND
Dried cannabis	0.14	0.24	ND	ND
Standard limit[2]	1.5	0.5	0.5	3.0

[1] Not detected; detection limit for As, Cd, Pb and Hg = 0.001 mg/kg
[2] Acceptable limits of heavy metal contaminants in cannabis provided by FDA

The cannabinoid extract (S18Th CO_2 extract) was purified by column chromatography to yield CBG)3.46(%, CBD)4.10(%, CBN)11.08(%, and THC) 12.96(%; the structures of these components were confirmed in previous reports [38] .The separation of Δ^9-THC and Δ^8-THC failed under the conditions used and the ^{1}H NMR showed peaks of Δ^9-THC and Δ^8-THC at a ratio of 1:1 .Thus, the mixture of THC was used for testing of its anticancer activities.

The tests for anticancer activities were performed on two types of cancer cell: HeLa cervical carcinoma cell and MDA-MB-231 (an epithelial human breast cancer cell), and two types of normal cells (LLC-MK2 and Vero cell lines). The results of testing are shown in Table 7. With the exception of CBG, all the test compounds possess notable anticancer properties. CBD selectively inhibits the MDA-MB-231 cell line and possesses strong anticancer activity against cancer cells with low cytotoxicity to LLC-MK2 normal cells. However, moderate cytotoxicity to Vero cells is observed. Although all cannabinoid extracts show lower anticancer activities than that of the anticancer drug doxorubicin, these cannabinoids should be further studied and the seized cannabis can be well used to develop the knowledge of their anticancer activity.

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Table 7. Anticancer activities of major components of cannabinoid extracts

Sample	IC_{50} (μg/mL)			
	HeLa	MDA-MB-231	LLC-MK2	Vero
Crude cannabis	9.97	na*	174.00	13.41
CBG	73.75	253.35	725.76	699.77
CBN	6.16	na	237.00	10.62
CBD	7.00	4.14	200.00	39.77
THC	10.00	16.49	204.00	67.23
Doxorubicin	1.85	1.50	98.92	99.48

* Not available

CONCLUSIONS

The study shows that the cannabis seized by the ONBC can be used for pharmaceutical and medicinal studies. Ethanolic extraction of the dry cannabis affords higher yields of the major components compared with the other methods although they are lower than other reported values due to the period and conditions of storage. The EO contains aromadendrene as the major terpene component. The levels of heavy metals in the dry plant were lower than the standard limits. Most extracts have CBD/Δ^9-THC value less than 1, causing psychotropic effects. Thus, they cannot be considered for pharmaceutical usage. The purification of the cannabis crude extract yields CBD (4%), CBG (3.46%), CBN (11.08%) and THC (12.96%).

The anticancer tests on the purified Hela and MDA-MB-231 cell lines reveal that CBD is the most active compound among the purified components. From the findings, the seized cannabis from ONBC can be used for research and development in the pharmaceutical and medical fields.

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