Pharkphoom PANICHAYUPAKARANANT https://orcid.org/0000-0003-1097-8822
Wiwit SUTTITHUMSATID https://orcid.org/0000-0001-6071-4644
Wanida SUKKETSIRI https://orcid.org/0000-0003-0836-1487

RESEARCH ARTICLE
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A Green Microwave-Assisted Extraction of C. *sativa* L. Extract and its Cytotoxic Activity Against Cancer Cells

PANICHAYUPAKARANANT et al. Green microwave-assisted extraction of Cannabis sativa

Pharkphoom PANICHAYUPAKARANANT[1], Wiwit SUTTITHUMSATID[1], Wanida SUKKETSIRI[2]

[1]Prince of Songkla University Faculty of Pharmaceutical Sciences, Department of Pharmacognosy and Pharmaceutical Botany, Hat-Yai, Thailand
[2] Prince of Songkla University Faculty of Science, Department of Pharmacology, Hat-Yai, Thailand

pharkphoom.p@psu.ac.th
orcid.org/0000-0003-1097-8822
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ABSTRACT

Objectives: This study aimed to explore the use of D-limonene and some vegetable oils with different amounts of saturated and unsaturated fatty acids as alternative green solvents for microwave-assisted extraction (MAE) of cannabis (*Cannabis sativa* L.). A standardized cannabis extract was selected to evaluate its potential as a chemopreventive agent.

Materials and Methods: Alternative green solvents, powder-to-solvent ratios, and irradiation cycles were determined to optimize the MAE conditions. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the cytotoxic effects against human breast cancer (MCF-7), liver cancer (HepG2), and mammary epithelium (hTert-HME1) cell lines.

Results: The extracts obtained from D-limonene and palm oil contained the highest concentrations of cannabidiol (CBD) and Δ-tetrahydrocannabinol (THC). A standardized D-limonene extract of cannabis (DEC) containing 0.03% w/w CBD and 1.37% w/w THC was selected for the evaluation of cytotoxic activity compared with CBD and THC. The results revealed that CBD and THC exhibited significant cytotoxic effects against MCF-7 and HepG2, with the IC_{50} values of 18.5 and 12.37 µg/mL for CBD and 24.21 and 4.30 µg/mL for THC, respectively, whereas DEC exhibited moderate cytotoxicity against MCF-7 (IC_{50} of 488.85 µg/mL). However, CBD and THC exhibited significant cytotoxicity against hTert-HME1 (IC_{50} values of 35.61 and 25.63 µg/mL, respectively), whereas DEC exhibited low cytotoxicity against hTert-HME1 (IC_{50} of 1.537.03 µg/mL).

Conclusion: DECs containing appropriate levels of THC and CBD have the potential to be candidates for cancer treatment. However, further investigations are required to improve the efficacy and safety profiles.

Keywords: Cannabis, cancer, limonene, microwave extraction, vegetable oil.

INTRODUCTION

Globally, cancer is a significant cause of morbidity and mortality, resulting in a large disease burden. According to Global Cancer Statistics 2020, breast cancer, with the largest number of 2.3 million new cases, accounted for 11.7% of all cancers, followed by lung cancer (11.4%), colorectal cancer (CRC) (10.0 %), while lung cancer was the main cause of cancer death (1.8 million deaths, 18%), followed by CRC (9.4%) and liver (8.3%) cancer.[1] Conventional treatments for cancer include surgery, chemotherapy, and radiotherapy. Although traditional treatments like

chemotherapy and radiotherapy are effective, they have limitations, such as severe side effects and the development of multidrug resistance in cancer cells. Medical cannabis is gaining attention as a treatment option for various diseases. Currently, the Food and Drug Administration has approved cannabis for specific conditions like nausea and vomiting, intractable epilepsy, and neuropathic pain. However, previous studies have explored the potential benefits of medical cannabis for various medical conditions, including cancer. Cannabis contains cannabinoids that interact with specific endogenous cannabinoid receptors, as well as other receptors, resulting in the expectation of anti-cancer effects.[2]

The cannabis industry now favors microwave-assisted extraction (MAE) because of its superior extraction efficiency compared to traditional methods.[3] Generally, organic solvents, including hexane, chloroform, and methanol, are commonly used for cannabis extraction.[3] However, most of these compounds are toxic to the human body,[4] which limits their industrial applications of these cannabis extracts. Therefore, the need for an alternative green solvent for cannabis extraction is a pressing concern because it can enhance the safety of the cannabinoid extraction process. Vegetable oils have higher cannabinoid content and a slower rate of cannabinoid degradation in cannabis extract than ethanol.[3] Furthermore, other natural compounds, such as D-limonene, are potential candidates primarily due to their nonpolar properties. D-limonene not only aids in the extraction process but also possesses inherent anti-cancer properties,[5] which may synergistically enhance the anti-cancer effects of the cannabis extract itself. This dual benefit supports the rationale for using D-limonene as an alternative green solvent.

This study aimed to investigate the potential of D-limonene and various vegetable oils, such as olive oil, sunflower oil, soybean oil, palm oil, and coconut oil, as green solvents for cannabis MAEs. The MAE conditions were optimized to yield cannabinoid-enriched extracts. Furthermore, we selected a standardized cannabis extract for cytotoxicity studies against human breast and liver cancers and compared it with normal human cells to assess its potential as a chemopreventive agent.

MATERIALS AND METHODS

Plant materials

Dried C. sativa inflorescences were obtained from the Faculty of Natural Resources, Prince of Songkla University, Thailand. The inflorescences were dried in a hot air oven and reduced to powder using an electric blender. The powder was then passed through a sieve to ensure its homogeneity.

Chemicals and materials

The purification and acquisition of tetrahydrocannabinol (THC) were accomplished using the method previously outlined.[6] The cannabidiol (CBD) compound was acquired from Chemfaces, a company based in Wuhan, China. Methanol, ethanol, and hexane were acquired from RCI Labscan (Bangkok, Thailand). D-limonene was procured from Krungthepchemi (Bangkok, Thailand). Sunflower oil, soybean oil, and palm oil were procured from Lam Soon (Thailand) Public Company situated in Samut Prakarn, Thailand. The acquisition of coconut oil was made from Ampol Food Processing, located in Nakornpathom, Thailand. The acquisition of olive oil was made from Sino-Pacific Trading, a company based in Bangkok, Thailand. The acquisition of rice bran oil was made from Oleen, a company in Samut Sakhon, Thailand. A Luna® C-18 column was obtained from Phenomenex (Bangkok, Thailand). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma Chemical, Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), modified Eagle's medium (MEM), and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell cultures

Human mammary epithelium (hTERT-HME1; ATCC CRL-4010™), human liver cancer (HepG2; ATCC HB-8065™), and human breast cancer (MCF-7; ATCC HTB-22™) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). hTERT-HME1 and HepG2 cells were cultured in DMEM, whereas MCF-7 cells were cultured in MEM. All cell lines were kept in a 5% CO_2 humidified incubator at 37°C. DMEM and MEM media were supplemented with 10% FBS, 1% 2 mM L-glutamine, 1% 100 IU/mL penicillin, and 100 µg/mL streptomycin.

Identifying alternative green solvents and MAE conditions

The powders of cannabis inflorescences were extracted with vegetable oils, D-limonene, ethanol, and hexane using MAE under the optimal conditions (for D-limonene and vegetable oils: microwave power: 900 W, irradiation time: 60 sec, and temperature: 115-120°C; for ethanol and hexane: microwave power: 450 W, irradiation time: 35 sec, and temperature: 65-70°C). The extracts were filtered, and the yields were recorded. The content of THC and CBD was determined using quantitative High performance liquid chromatography (HPLC). The cannabis powders were subsequently extracted with suitable solvents using MAEs with different powder-to-solvent ratios and irradiation cycles. The extracts were then filtered and the yields were recorded. The content of THC and CBD was determined using quantitative HPLC. All experiments were performed in triplicate.

Quantitative HPLC of THC and CBD

We used the previously described HPLC method with some modifications to determine the content of CBD and THC in the cannabis extracts. Briefly, the analysis was performed using an UFLC Shimadzu model equipped with a photodiode-array detector and autosampler (Shimadzu, Japan) at a wavelength of 220 nm. A 4.6 mm×250 mm, 5 µm Luna® C18 column (Phenomenex, Thailand) was eluted with a mobile phase consisting of 85% v/v methanol in water at a flow rate of 1 mL/min.

Calibration curves for THC and CBD were established using six concentrations (from 6.25 to 200 µg/mL. based on linear regression, the calibration curves of CBD and THC were Y=72615X+72146 (r^2 = 0.9998) and Y=54467X+77267 (r^2 = 0.9999), respectively.

The samples (2.0 mg) were accurately weighed and diluted with methanol to 10 mL in a volumetric flask. Prior to HPLC analysis, the sample solutions were filtered through a 0.45 µm membrane filter. The experiments were performed in triplicate.

Determination of anticancer activity

Anti-cancer activity was determined using the MTT assay.[7] Briefly, HepG2, MCF-7 and hTERT-HME1 cells were seeded into a 96-well microplate at a density of 1×10^4 cells per well and then incubated in a 5% CO_2 humidified incubator at 37°C for 24 h. The cells were treated with sample solutions at various concentrations, including CBD (3.12, 6.25, 12.5, 25, and 50 µg/mL), THC (3.12, 6.25, 12.5, 25, and 50 µg/mL), DEC (125, 250, 500, 1000, and 2000 µg/mL), D-limonene (125, 250, 500, 1000, and 2000 µg/mL), and 5-FU (1, 5, 10, 50, and 100 µg/mL), and then incubated at 37°C for 24 h. The media was removed, and the cells were treated with MTT solution (500 µg/mL) and incubated for 2 h at 37°C. The formazan product was solubilized with DMSO, and the intensity of solutions was measured at 570 nm using a microplate reader (Biotek, Winooski, VT, USA). 5-Fluorouracil (5-FU) was used as a positive control. The percentage of cell viability relative to non-treated cells was presented as a negative control. The selectivity index (SI) was calculated by dividing the IC_{50} values of the samples by those of cancer and normal cells.

Statical analysis

The results are expressed as the mean ± standard deviation. A statistically significant difference was evaluated using one-way analysis of variance, followed by Duncan's multiple range test (p<0.05).

RESULTS

Identifying an alternative solvent for extraction

This study determined D-limonene and some vegetable oils containing different ratios of unsaturated to saturated fatty acids (SFAs) as alternative green solvents for the extraction of THC and CBD from *C. sativa* inflorescences using the MAE method and compared them to conventional solvents such as ethanol and hexane. Based on an HPLC analysis (Figure 1), ethanol and hexane provided the extracts with the highest cannabinoid concentrations, especially the THC levels (Table 1). The results of this study indicate that D-limonene, coconut oil, and palm oil have nonpolar properties similar to those of CBD and THC. As a result, they can be an alternative green solvent to extract cannabinoids. However, for nutraceutical applications, coconut oil consumption considerably increases the levels of low-density lipoprotein cholesterol and total cholesterol compared with palm oil, which may increase the risk of cardiovascular disease.[8] Therefore, D-limonene and palm oil are considered suitable alternative green solvents for cannabinoid extraction, with the aim of producing functional food products.

Optimization of the extraction conditions

This study evaluated the effects of varying amounts of cannabis powder (1, 2, 4, and 6 g) per 20 mL of solvent extracted using the MAE method. The results revealed that a powder-to-solvent ratio of 4 g per 20 mL produced the cannabis extracts with the highest total yields of CBD and THC for palm oil and D-limonene (Table 2). Notably, the concentrations of both THC and CBD increased as the powder content increased. However, the extraction yields of the extracts were markedly reduced at ratios greater than 4 g per 20 mL due to solvent adsorption by cannabis powders, which resulted in a decrease in total yields of both THC and CBD. Moreover, in this study, the irradiation cycles up to three cycles (one cycle was 70 sec power-on and 50 sec power-off) resulted in a significant increase in total yields of THC and CBD for both palm oil and D-limonene cannabis extracts (Table 3). These MAE conditions increased the extraction temperature to 110°C. In contrast, increased irradiation cycles of more than 3 cycles, which resulted in a higher extraction temperature, did not significantly increase the total yields of cannabinoids in either extract.

Determination of the anticancer activity of cannabinoids

As shown in Table 4, the MTT assay revealed that both THC and CBD had strong cytotoxicity against MCF-7 and HepG2. They also had strong cytotoxicity against HTERT-HME1. DEC containing 0.03% w/w CBD and 1.37% w/w THC exhibited moderate cytotoxicity against MCF-7 and low cytotoxicity against HepG2. However, DEC exhibited very low cytotoxicity against hTERT-HME1. On the other hand, D-limonene demonstrated reduced cytotoxic effects on MCF-7 cells and showed no cytotoxicity toward HepG2 and hTERT-HME1.

DISCUSSION

The superior efficiency and environmental benefits of MAE make it suitable for extracting cannabinoids from cannabis. Compared with conventional methods such as heat reflux extraction, soxhlet extraction, supercritical fluid extraction, and ultrasound-assisted extraction, MAE consistently achieves the highest extraction yields of cannabis cannabinoids.[9] This method is more effective and sustainable, requires significantly less solvent and requires a shorter time frame. These advantages make MAE an optimal choice for cannabinoid extraction.

According to reports, THC and CBD are nonpolar compounds that display nearly identical lipophilicity, with log P values of 5.41 and 5.42, respectively. However, their water solubility differed slightly, with log S values of 5.93 and 5.41, respectively.[10] Therefore, nonpolar solvents should extract both CBD and THC with high efficiency. Based on the chemical structures of D-limonene and vegetable oils, which exhibit nonpolar properties, they can be used as an alternative green solvent for extracting naturally occurring active compounds with nonpolar properties. The major component of vegetable oils is triglycerides, which are esters of fatty acids and glycerol. Different types of fatty acid composition affect the physical and chemical properties of triglycerides, resulting in different extraction efficiencies for vegetable oils.[11] For example, coconut oil and palm oil contain higher levels of SFAs than the others. The major SFA in coconut oil is lauric acid, whereas palm oil contains palmitic acid as the predominant SFAs.[12] However, among the alternative solvents, D-limonene, palm oil, and coconut oil produce the highest total yields of cannabinoid content, which are not significantly different from those of the ethanol and hexane extracts. Because ethanol and hexane have a low boiling point, they are highly volatile in the MAE. Thus, although these two solvents produced the extract with higher concentrations of both cannabinoids, they produced lower extraction yields and, therefore, produced slightly lower total yields of cannabinoids than those extracted from D-limonene, palm oil, and coconut oil.

In addition to solvent polarity, powder-to-solvent ratios, and microwave irradiation cycles are common factors that affect MAE efficiency. According to mass transfer principles, during the solid-liquid extraction process, the powder-to-solvent ratio has a significant impact on the concentration gradient between the solute in the powder and the solvent at the surface of the raw material.[13] The increasing diffusion rate of the compounds from the extracted powder into the solvent depends on the concentration gradient, which increases with increasing powder-to-solvent ratio. However, the concentration gradient does not continue to increase once equilibrium is reached, which is characterized by the relationship between the amount of powder and solvent used that gives the maximum yields.[13,14] Additionally, researchers typically perform the MAE method under several irradiation cycles to prevent overheating or bumping during herbal extraction. Furthermore, the number of irradiation cycles in MAE has a significant impact on extraction time and temperature. Basically, time and temperature are critical extraction conditions because they affect the solubility, mass transfer, and stability of natural compounds. However, prolonged extraction and extreme temperatures may lead to the degradation of bioactive compounds.[14]

Recent reports indicate that D-limonene inhibits anti-cancer activity through various mechanisms of action.[15] Accordingly, cannabis extraction using D-limonene has attracted attention and has the potential to be a novel anticancer nutraceutical. The cytotoxicity categorization[16] classifies DEC as having moderate cytotoxicity (IC_{50}: 100-500 g/mL, for herbal extract) against MCF-7 and THC and CBD as potentially toxic substances with moderate cytotoxic activity (IC_{50}: 20-100 M, for pure compounds) against MCF-7 and HepG2 cell lines. However, only THC exhibited very strong cytotoxicity against HepG2 cell lines (IC_{50}: 1-20 μM, for the pure compound). Calculating the SI value is crucial for evaluating the anticancer activity of herbal drugs. A SI value>3 is classified as a prospective anti-cancer sample.[17] According to these standards, the SI data in Table 4 showed that DEC was specifically toxic to MCF-7 cells, whereas CBD and THC were not selectively toxic to cells. Although DEC contained only 1.37% w/w of THC and 0.03% w/w of CBD, it also showed potential cytotoxicity against MCF-7 with higher selectivity than CBD and THC. Nevertheless, using D-limonene as an alternative green solvent for the preparation of a cannabis extract may improve its anti-cancer effects. However, the enhancement of DEC's anticancer properties necessitates careful consideration of cannabis strain selection, specifically those characterized by an ideal balance between THC and CBD. This critical factor plays a pivotal role in the production of cannabis extracts with maximized anticancer potential.

CONCLUSION

The present study identified D-limonene and palm oil as promising alternative green solvents for extracting cannabinoids from cannabis inflorescences under MAE optimal conditions. The MAE method offers a number of advantages, including reduced time and energy consumption. In this study, DEC exhibited moderate cytotoxicity against MCF-7 cells with higher selectivity than CBD and THC. Therefore, DEC containing an appropriate amount of THC and CBD may exhibit a more satisfying anticancer effect and be a promising candidate for cancer treatment. However, additional research is required to understand the mechanisms of anticancer activity and to investigate additional efficacy and safety profiles.

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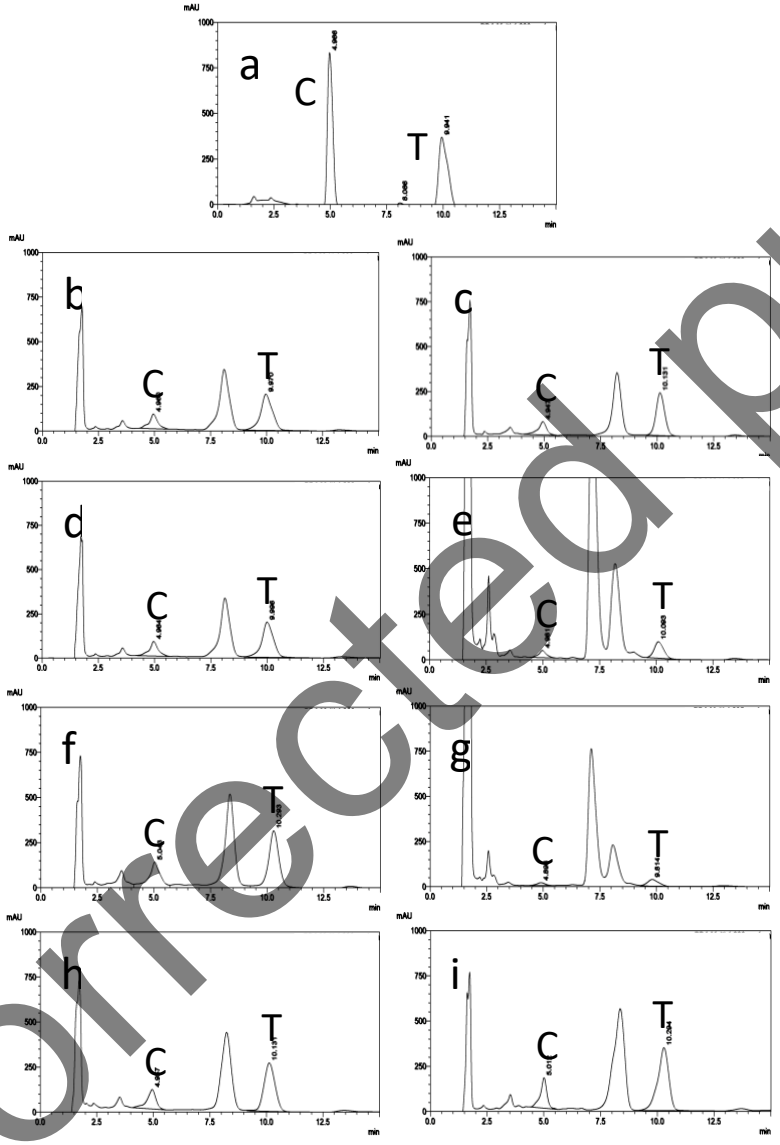

Figure 1. High performance liquid chromatography chromatograms of the cannabidiol and tetrahydrocannabinol standard (a) and cannabinoids extracts using sunflower oil (b), olive oil (c), soybean oil (d), palm oil (e), coconut oil (f), D-limonene (g), ethanol (h), and hexane (i) as the extraction solvents

Table 1. Cannabinoid content and yields of *C. sativa* inflorescence extracts obtained using various extraction solvents

Solvents	Mean±SD				
	Extraction yield (mL)	CBD concentration (mg/mL)	THC concentration (mg/mL)	Total yield of CBD (mg/2 g powders)	Total yield of THC (mg/2 g powders)
Sunflower oil	11.83±0.14^{a}	0.04±0.00^{a}	2.04±0.19^{a,b}	0.45±0.02^{a}	24.24±2.47^{a}
Olive oil	12.75±0.50^{a}	0.04±0.00^{a}	1.87±0.12^{a}	0.51±0.01^{a}	23.81±0.71^{a}
Soybean oil	12.08±0.88^{a}	0.05±0.00^{b}	2.01±0.12^{a,b}	0.62±0.04^{b}	24.32±0.70^{a}
Palm oil	13.08±0.52^{a}	0.06±0.01^{c}	2.25±0.06^{b,c}	0.77±0.07^{c,d}	29.45±1.32^{b}
Coconut oil	12.33±1.26^{a}	0.06±0.00^{c}	2.43±0.02^{c}	0.73±0.08^{c}	29.96±3.10^{b}
D-limonene	12.67±0.29^{a}	0.07±0.00^{d}	2.34±0.05^{b,c}	0.85±0.05^{d}	29.61±0.73^{b}
Ethanol	9.75±0.87^{b}	0.05±0.00^{b}	2.99±0.46^{d}	0.50±0.06^{a}	28.85±2.21^{b}
Hexane	8.33±0.38^{c}	0.09±0.00^{e}	2.98±0.07^{d}	0.71±0.05^{c}	24.82±1.73^{a}

Values with non-identical letters in the same column are significantly different with statistic values $p<0.05$. Total yield of cannabinoids=extraction yield (mL)×concentration (mg/mL). CBD: Dannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation

Table 2. Effects of powder-to-solvent ratios on CBD and THC content and yields of *C. sativa* inflorescence extracts containing palm oil and D-limonene

Solvent	Powder content (g)	Mean±SD				
		Extraction yield (mL)	CBD conc. (mg/mL)	THC conc. (mg/mL)	Total yield of CBD (mg)	Total yield of THC (mg)
Palm oil	1	14.50±0.43^{a}	0.02±0.00^{a}	1.03±0.02^{a}	0.35±0.03^{a}	15.23±0.38^{a}
	2	13.08±0.14^{b}	0.06±0.01^{b}	2.13±0.06^{b}	0.74±0.03^{b}	28.26±0.83^{b}
	4	8.75±0.66^{c}	0.08±0.01^{c}	4.10±0.12^{c}	0.67±0.08^{b,c}	34.87±1.03^{c}
	6	4.92±0.38^{d}	0.11±0.01^{d}	6.41±0.26^{d}	0.56±0.03^{c}	27.60±1.32^{d}
D-limonene	1	14.75±0.25^{a}	0.01±0.00^{a}	0.63±0.05^{a}	0.14±0.00^{a}	9.07±0.74^{a}
	2	13.18±0.16^{b}	0.11±0.01^{b}	2.13±0.02^{b}	1.34±0.17^{b}	27.18±0.30^{b}
	4	8.42±0.52^{c}	0.27±0.01^{c}	5.51±0.11^{c}	2.18±0.07^{c}	44.11±0.88^{c}
	6	5.25±0.25^{d}	0.34±0.00^{d}	7.19±0.10^{d}	1.63±0.01^{b}	33.74±0.45^{d}

Values with non-identical letters in the same column differ significantly for each solvent (statistical values $p<0.05$. Total yield of cannabinoids=extraction yield (mL)×concentration (mg/mL).
CBD: Cannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation.

Table 3. Effects of irradiation cycles on CBD and THC content and yields of *C. sativa* inflorescence extracts containing palm oil and D-limonene

Solvent	Irradiation cycle	Mean±SD				
		Extraction yield (mL)	CBD conc. (mg/mL)	THC conc. (mg/mL)	Total yield of CBD (mg/2 g powders)	Total yield of THC (mg/2 g powders)

Palm oil	0.5	12.40±0.53[a]	0.04±0.00[a]	1.74±0.06[a]	0.53±0.04[a,b]	22.59±0.83[a]
	1	12.23±1.08[a]	0.04±0.00[a]	1.81±0.03[a]	0.56±0.03[b,c]	23.53±0.37[a]
	2	12.08±0.14[a]	0.04±0.01[a]	1.87±0.10[a]	0.49±0.06[a]	22.43±1.14[a]
	3	12.68±0.08[a]	0.04±0.00[a]	2.11±0.08[b]	0.52±0.02[a,b]	26.93±1.00[b]
	4	12.47±0.06[a]	0.04±0.00[a]	2.15±0.07[b]	0.55±0.02[a,b,c]	26.84±0.86[b]
	5	12.54±0.46[a]	0.05±0.00[a]	2.15±0.04[b]	0.60±0.01[c]	26.82±0.46[b]
D-limonene	0.5	12.65±0.37[a]	0.06±0.00[a]	2.08±0.11[a]	0.73±0.01[a]	27.00±1.39[a]
	1	12.24±0.28[a]	0.06±0.00[b]	2.22±0.01[a,b]	0.81±0.01[b]	27.81±0.27[a,b,c]
	2	12.30±0.09[a]	0.07±0.00[c]	2.25±0.05[b]	0.81±0.01[b]	27.51±0.57[a,b]
	3	12.67±0.10[a]	0.07±0.00[d]	2.35±0.02[b]	0.87±0.01[c]	29.39±0.21[d]
	4	12.65±0.32[a]	0.07±0.00[c]	2.24±0.07[b]	0.86±0.01[c]	29.14±0.91[c,d]
	5	12.70±0.60[a]	0.07±0.00[c]	2.21±0.03[a,b]	0.86±0.01[c]	28.75±0.01[b,c,d]
Values with non-identical letters in the same column differ significantly for each solvent (statistical values $p<0.05$. Total yield of cannabinoids=extraction yield (mL)×concentration (mg/mL). CBD: Cannabidiol, THC: Tetrahydrocannabinol, SD: Standard deviation.						

Table 4. Cytotoxic activities of DEC, CBD, and THC against MCF-7 and HepG2 cancer cells and hTERT-HME1 normal cells

Compounds	IC_{50} (µg/mL)			Selectivity index	
	hTERT-HME1	MCF-7	HepG2	MCF-7	HepG2
CBD	35.61	18.46	12.37	1.93	2.88
THC	25.63	24.21	4.30	1.06	5.96
DEC	1537.03	488.85	1336.97	3.14	1.15
D-limonene	N.A.	1150.9	n/a	n/a	n/a
5-FU	n.a.	1.9	99.83	n/a	n/a

hTERT-HME1: human mammary epithelium, MCF-7: human breast cancer cells, HepG2: human liver cancer, DEC: standardized D-limonene extract of cannabis, IC_{50}: 50% inhibitory concentration, n/a: not active at 2000 µg/mL for D-limonene and 100 µg/mL for 5-FU, n/a: not available data for selectivity index.
CBD: cannabidiol, THC: tetrahydrocannabinol.