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Ultrasound-Assisted Extraction of Cannabidiol from Moroccan *Cannabis sativa* L. (Beldia) and Antioxidant Activities of Its Fractions †

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Abstract: Cannabidiol (CBD), a major phytocannabinoid in *Cannabis sativa*, exhibits diverse therapeutic properties, as demonstrated by in silico, in vitro, and in vivo studies. These properties include cardioprotective, analgesic, anti-inflammatory, antioxidant, antitumor, neuroprotective, and anticancer effects. This study describes the ultrasound-assisted extraction, isolation, and characterization of CBD as a major product from Moroccan *Cannabis sativa* resin. The petroleum ether-dichloromethane (PE-DI), methanol, and water were used as extracting solvents by increasing gradient polarities. Isolation of CBD was achieved through successive normal silica and reversed-phase RP18 silica gel column chromatography of the PE-DI fraction (7:3). The characterization was conducted using infrared (IR) and nuclear magnetic resonance (NMR) techniques. The antioxidant activities of fractions were assessed by the DPPH and FRAP assays. Total phenolic and total flavonoid contents were measured with the Folin-Ciocalteu reagent and aluminum trichloride methods, respectively.

Keywords: *Cannabis sativa*; cannabidiol; antioxidant assays; phenolic content; flavonoid content

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1. Introduction

Plants have long played a key role in human life. They are used for nourishment, defense, and treatment. More broadly, cannabis has therapeutic effects such as antioxidant, antibacterial, anti-inflammatory, and enzyme-inhibiting properties due to the presence of cannabinoids, terpenoids, flavonoids, and alkaloids [1]. Cannabis is a type of medicinal plant with three species *sativa*, *indica*, and *ruderalis*. Cannabis contains over 144 compounds known as cannabinoids, of which the most well-known are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC). *Cannabis sativa* has been used for medicinal purposes to manage chronic pain and also to reduce inflammation and treat anxiety disorders [2].

Cannabidiol (CBD), one of the major phytocannabinoids in *Cannabis sativa*, exhibits diverse therapeutic properties, as demonstrated by in silico, in vitro, and in vivo studies [3,4]. These properties include antiseizure, anticonvulsant, antiarthritic, cardioprotective, analgesic, anti-inflammatory, antioxidant, antitumor, neuroprotective, antiepileptic, and anticancer effects [5,6]. It is in this context that *Cannabis sativa*, a medicinal plant that is widely distributed in the North of Morocco, has been chosen for isolation of CBD and antioxidant investigations. Antioxidant activities have gained increasing interest due to the important role played by antioxidant compounds in treating and preventing diseases linked to oxidative stress [7].

This study aims to optimize ultrasound-assisted extraction of bioactive compounds from the resin of Moroccan *Cannabis sativa* (Beldia), isolate CBD, and evaluate the antioxidant activities of fractions using DPPH and FRAP assays.

2. Material and Methods

2.1. Plant Material and Extraction

The resin of Cannabis sativa was collected from Ketama in the North of Morocco in March 2024. The plant matériel was carried to the Euromed University of Fez for further treatment and analysis. The Cannabis sativa resin (44.64 g) powder was placed in Erlenmeyer flasks and a petroleum ether-dichloromethane mixture (PE-DI 7:3) was added. The erlenmeyer was then placed in an ultrasonic bath (30 °C, 240 W, 45 kHz, VWR USC, Germany) for 60 min to extract the bioactive compounds. After filtration, methanol was added to the residue followed by distilled water. The extracts were carefully dried and stored at 4 °C for further analysis.

2.2. Extraction Procedure

To extract bioactive compounds from Cannabis sativa resin, four solvents have been used petroleum ether, dichloromethane, methanol, and water.

2.2.1. Petroleum Ether-Dichloromethane Extraction (PE-DI)

The resin (44.64 g) was mixed with 300 mL of PE-DI mixture (7:3) and placed in an ultrasonic bath for one hour. The process was repeated twice to extract the maximum of bioactive compounds. After filtration, the filtrate was dried using rotavapor under reduced pressure. The dried extract was weighed using the analytical balance and stored at 4 °C for further analysis.

2.2.2. Methanol Extraction

The residue from the PE-DI extraction was mixed with 300 mL of methanol, and placed in an ultrasonic bath for one hour (the process was repeated twice). After filtration, the filtrate was dried using rotavapor under reduced pressure. The dried extract was weighed using the analytical balance and stored at 4 °C for further analysis.

2.2.3. Water Extraction

The residue from the methanol extraction was treated with 300 mL of distilled water, and the same protocol as described for methanol extraction was followed.

2.3. Determination of Extraction Yield

To determine the extraction yield of each solvent, the Equation (1) has been used:

$$\textbf{Yield (\%) = weight of dried extract/weight of starting plant material × 100} \quad (1)$$

2.4. Determination of the TPC, TFC, and Antioxidant Assays

The total phenolic content (TPC) in the fractions was assessed using the Folin-Ciocalteu method, as described by Lawag et al. [8], while the total flavonoid content (TFC) was determined using the aluminum chloride colorimetric assay outlined by Magalhaes et al. [9]. The antioxidant activity was assessed through two assays using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays. The free radical scavenging potential was evaluated following the method reported by Medini et al. [10]. Finally, the ferric-reducing antioxidant power (FRAP) assay was performed using a modified version of the method described by Govindappa et al. [11].

2.5. Column Chromatography

The column chromatography technique has been used to purify the PE-DI fraction. The purification was performed on Merck silica gel 60 μm (215–400 mesh), and reversed-phase RP18 silica gel 90. Analytical thin-layer chromatography (TLC) was performed on Sigma-Aldrich aluminum plates coated with silica gel 60 F_{254} (thickness 0.2 mm). The products were visualized by UV lamp at 254 and 365 nm. Several solvents have been used to purify CBD, including n-hexane, dichloromethane, acetone, methanol, and 0.5% acetic acid in distilled water

2.6. NMR and IR Analyses

The ^{1}H-NMR spectrum of isolated CBD was acquired using a Joel AC 600 MHz NMR spectrometer (Jeol, Tokyo, Japan). The infrared spectrum was recorded on a Nicolet IS50 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) at room temperature. The spectroscopic data of CBD were compared to those from the literature.

3. Results

3.1. Extraction Yield

Extraction of bioactive compounds from the resin of Moroccan Cannabis sativa yielded 51.38, 21.43, and 2.18% for PE-DI, methanol, and water respectively. Therefore, the final extraction yield reached 74.99%, indicating a solid extraction efficiency.

3.2. Separation and Purification of CBD Using Column Chromatography

A portion of the PE-DI fraction (22.00 g) was subjected to column chromatography separation, packed with normal silica gel in the gradient of increasing polarities. The mixture of three solvents (n-hexane, dichloromethane, and acetone) was used as an elution system in the ratios of 10:0.3:0.3 to 10:5:5. Based on TLC analysis, the 51 subfractions collected were combined to get 11 subfractions.

To purify the compounds from the subfraction 4 (3.52 g), reversed-phase RP18 column chromatography has been used. The mixture of three solvents (methanol, 0.5% acetic acid in distilled water, and dichloromethane) was used as an elution system in the ratio of 6:2:2 in isocratic mode. Thirty-eight (38) subfractions have been collected and the recrystallization of subfraction 1 in cooled n-hexane leads to pure CBD (Figure 1).

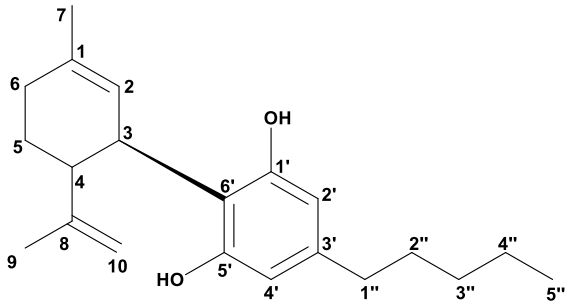

Figure 1. Chemical structure of isolated CBD.

Cannabidiol, IR (ν_{max} cm^{-1}): 3421 (OH stretch), 2924 CH and 2855 [C(sp^3)-H stretch], 1622, 1579, and 1442 (C=C, stretch), 1026 (C-O-C, stretch). ^{1}H-NMR (600 MHz, $CDCl_3$, ppm) δ: 6.63 (1H, s, H-2′); 6.24 (1H, s, H-4′); 5.56 (1H, m, H-2); 4.51 (2H, m, H-10); 4.08 (1H, d, J = 9.9 Hz, H-3); 2.91 (1H, m, H-4); 2.42 (2H, t, J = 6.0 Hz, H-1″); 2.21 and 2.11 (2H, m, H-6); 1.78 (2H, m, H-5); 1.70 (3H, s, H-7); 1.64 (3H, s, H-9); 1.55 (2H, m, H-2″); 1.32 (4H, m, H-3″ and H-4″); and 0.88 (3H, t, J = 6.0 Hz, H-5″). These IR and ^{1}H-NMR data were compared with those of CBD from the literature [12].

3.3. Determination of TFC, TFC, DPPH Assay, and FRAP Assay

Gallic acid and quercetin were used as standards in TFC and TFC determinations respectively. Ascorbic acid was used as a standard in the FRAP assay; while in the DPPH assay, standards were ascorbic acid and Butylated hydroxytoluene (BHT). The results of all these analyses are presented in Table 1 below.

Table 1. Results of TFC, TFC, DPPH assay, and FRAP assay.

Extracts	TPC in mg EAG/g	TFC in mg QE/g	DDPH (IC50 in μg/mL)	FRAP (IC50 in μg/mL)
PE-DI fraction	44.91	3.73	62.54	50.48
Methanol fraction	28.78	2.14	96.12	12.95
Aqueous fraction	5.91	0.17	252.72	204.97
Ascorbic acid	-	-	7.09	5.23
BHT	-	-	33.61	-

4. Discussion

Several studies have shown that extracts obtained using different solvents have variable biological activities [13,14]. Consequently, the appropriate extraction technique and solvent must be defined according to the quality of the sample matrix and the activities desired [15]. In this study, the extraction efficiency results reflect the effectiveness of the process, suggesting its potential for further applications in the extraction and isolation of bioactive compounds from *Cannabis sativa*. Indeed, successive extraction starting with the less polar solvent to the more polar solvent enabled maximum extraction of bioactive compounds, leading to a final yield of 74.99%.

The TPC in the PE-DI fraction was the highest (44.91 mg EAG/g) among the three fractions, followed by the methanol fraction with a TPC of 28.78 mg EAG/g. The aqueous fraction showed the lowest TPC with 5.91 mg EAG/g. These values are within the range of total polyphenol content in cannabis extracts found by other researchers. In the study conducted by Izzo et al. [16], they found values ranging from 10.51 to 52.58 mg GAE/g of extract, depending on the extraction method used.

Concerning quantification of TFC, the PE-DI fraction had the highest total flavonoid content (3.733 mg QE/g), followed by the methanol fraction with a TFC of 2.14 mg QE/g. The aqueous fraction showed the lowest TFC with 0.17 mg QE/g. In the study carried out by Elsohly et al. [17], they reported flavonoid content in *Cannabis sativa* ranging from 0.5 to 2 mg QE/g of plant material, depending on the extraction method used. The PE-DI fraction from our study demonstrated relatively high flavonoid content with the TFC of 3.733 mg QE/g. This highlights the potential of PE-DI fraction for further applications in medical and nutritional fields due to their high flavonoid content.

In the DPPH assay, ascorbic acid and Butylated hydroxytoluene (BHT) were used as standards. The IC_{50} of standards were compared to the IC_{50} of PE-DI, methanol, and aqueous fractions. The IC_{50} of the PE-DI fraction (IC_{50} = 62.54 μg/mL) was less potent compared to the IC_{50} of standards (7.09 and 33.61 μg/mL). The methanol fraction showed an IC_{50} of 96.12 μg/mL, while the aqueous fraction had the highest IC_{50} of 252.72 μg/mL compared to the other fractions and standards. The study carried out by Cásedas et al. [18] reported IC_{50} values for various extracts of *Cannabis sativa* ranging from 60 to 127 μg/mL, depending on the extraction method and the specific type of extract. The methanol fraction demonstrated moderate antioxidant potential, while the PE-DI fraction showed slightly better antioxidant activity. However, the aqueous fraction showed significantly lower antioxidant activity.

For the FRAP assay, the methanol fraction (IC_{50} = 12.95 μg/mL) demonstrated effective antioxidant activity, compared to PE-DI fraction (IC_{50} = 50.48 μg/mL), but was less effective when we compared it to ascorbic acid (IC_{50} = 5.23 μg/mL). The IC_{50} of aqueous

fraction (IC_{50} = 204.97 μg/mL) showed a high value of IC_{50} compared to IC_{50} of PE-DI fraction, methanol fraction, and ascorbic acid. Cásedas et al. [18] reported FRAP IC_{50} values for different *Cannabis sativa* extracts ranging from 15 to 90 μg/mL which shows that the found values in this study are in range. These results showed that the methanol fraction has a higher antioxidant potential than the PE-DI and aqueous fractions. The aqueous fraction, with its high IC_{50} value, showed the lowest antioxidant activity, underscoring the fact that the solvents used previously extracted the maximum amount of compounds with antioxidant properties.

Concerning the isolation of CBD, IR and ^{1}H-NMR were used to confirm its chemical structure. The peaks at δ 6.63 and 6.24 ppm correspond to aromatic protons H-2′ and H-4′ respectively, which are characteristics of CDB, and the signal at δ 5.56 ppm corresponds to the ethylenic proton H-2 of DBD. Peaks between 1.55–0.88 ppm are attributed to the alkyl chain of CBD. The ^{1}H-NMR data of CBD were compared with those published in the literature for confirmation of the chemical structure [12,19].

5. Conclusions

In summary, the ultrasound-assisted extraction yield was highest for the PE-DI fraction (51.38%), followed by the methanol fraction (21.43%), and lowest for the aqueous fraction (2.18%), resulting in a total extraction yield of 74.99%. Using column chromatography, CBD has been isolated and characterized through its IR and NMR data. The DPPH and FRAP assays have been used for antioxidant assessment and the results demonstrated that PE-DI and methanol fractions exhibited significantly greater antioxidant activities than aqueous fraction.

Future research will focus on the isolation and characterization of the compounds present in other subfractions, as well as evaluating the antimicrobial properties of both the fractions and isolated compounds through in vitro and in vivo studies.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure 2: IR spectrum of CBD; Figure 3: ^{1}H-NMR spectrum of CBD.

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