Acta Biologica Marisiensis sciendo

INDIGENOUS *CANNABIS SATIVA* - THE PHARMACOGNOSTICAL ANALYSIS OF A WEED-TYPE VARIETY

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Received: 16 November 2023; **Accepted:** 8 January 2024; **Published:** 30 June 2024

Abstract: *Cannabis sativa* is a well-known plant that has attracted a lot of attention because of its remarkable therapeutic potential. However, a variety of this species is growing invasive in some cultures in Romania. This variety has a different composition compared to the cultivated variety and a notably low Δ9-tetrahydrocannabinol (THC) content, making the occurrence of psychoactive effects highly improbable. This research aimed to evaluate from a pharmacognostical point of view the herbal drug collected from Romania. A preliminary microscopic analysis was performed to confirm the identity of the herbal drug. A qualitative analysis by thin-layer chromatography was performed to identify cannabinoids and flavonoids. Quantitative determinations included the evaluation of total polyphenolic content, total flavonoid content, and hydroxycinnamic acid content, as well as ion chromatographic analysis for anion quantification. Antioxidant and alpha-amylase activities were performed as well. The results indicated that the herbal drug contains only moderate concentrations of polyphenolic compounds, but has a good antioxidant activity, likely attributable to the presence of non-polyphenolic compounds. Moreover, the extract had a moderate alpha-amylase inhibitory activity, suggesting a low potential in nutritional and therapeutic contexts.

Keywords: *Cannabis sativa*, invasive species, cannabidiol, polyphenols, antioxidant activity

1. Introduction

Cannabis sativa L. is cultivated in Romania for its fibers that are used in the textile industry. The cultivated species (encoded NC 5302 10 00) must have a Δ9-tetrahydrocannabinol (Δ9-THC) level below 0.3%, and the seeds must be accompanied by documents that attest the level of THC, according to the Regulation (EU) no 1308/2013

of the European Parliament and of the Council of 17 December 2013. In Romania, *Cannabis sativa* is included in the list of plants dangerous for human consumption (*ORDIN 244 22/04/2005 - Privind Prelucrarea, Procesarea Şi Comercializarea Plantelor Medicinale Şi Aromatice Utilizate ca Atare, Parţial Procesate Sau Procesate Sub Formă de Suplimente Alimentare Predozate*, n.d.). According to Law no. 339/2005 regarding the legal regime of narcotic and psychotropic plants, substances, and preparations, tetrahydrocannabinol is included in the list of substances without recognized interest in medicine. *Cannabis, cannabis* resin, extracts, and tinctures from this plant are included in the category of 'Other plants, substances, and preparations containing narcotic and psychotropic substances of medical interest, subjected to strict control', according to the same law (*LEGE 339 29/11/2005 - Privind Regimul Juridic al Plantelor, Substanţelor Şi Preparatelor Stupefiante Şi Psihotrope*, n.d.).

Over time there have been many disputes regarding the nomenclature of *Cannabis* species. A two step classification system has been used in the twenty years, which was initially proposed by Cronquist and Small. The first step is represented by the THC content (a value of 0.3% is the dividing point). Based on the THC content there are two varieties of *Cannabis sativa: Cannabis sativa* subsp. *sativa* (low THC content), and *Cannabis sativa* subsp. *indica* (high THC content). The second step proposed by the two authors is related with the domestication characteristics of the subspecies: *Cannabis sativa* subsp. *sativa* var. *sativa* having domestication characteristics, *Cannabis sativa* subsp. *sativa* var. *spontanea* having the characteristics of a wild population, *Cannabis sativa* subsp. *indica* var. *indica* having the characteristics of a cultured variety, and C*annabis sativa* subsp. *indica* var. *kafiristanica* having the characteristics of a wild population. They also have declared that it is quite difficult to distinguish wild populations from the cultivated ones, due to the hybridization between these populations. Height is usually a less important characteristic in the identification process of the species, because large variations were seen due to the geographical conditions. The morphology of the fruit can offer information about the origin of the *Cannabis* variety. The perianth is present in the wild populations, while in the cultivated populations, the perianth is often missing at maturity. However since the early 1970's and until now, the classification of *Cannabis species* is still on debate (McPartland, 2018; McPartland and Small, 2020; Small and Cronquist, 1976).

Cannabinoids are benzopyranic derivatives, terpenophenolic compounds having 21 carbon atoms in the molecule. In the freshly harvested material, they are found mostly in the form of acids. The percentage of cannabinoids in the herbal drug can vary from 2 to 20%, depending on the variety and the growing conditions (Evans, 2009; Mechoulam and Hanuš, 2000). The herbal drug also contains a percentage of up to 0.01% of spermidine alkaloids (Mechoulam, 1989; Radwan et al., 2021). At the same time, there is also a percentage of up to 0.5% of essential oil, consisting of over 120 constituents. The volatile compounds are mainly monoterpenes and sesquiterpenes, such as: α- and β-pinene, myrcene, limonene, α-bergamotene, α-caryophyllene and β-caryophyllene (Aizpurua-Olaizola et al., 2016, Vasudevan and Sthal, 2020). Also, other classes of substances, such as flavonoids, have been identified in the *Cannabis sativa* species. The most common flavonoids in hemp are flavones (mainly apigenin and luteolin) and flavonols (quercetin) derivatives, while cannflavins A, B, and C are mostly specific to this plant. Based on their

chemical structures, cannflavins A and B can be derived from luteolin (Bautista et al., 2021).

The primary objective of this study was to conduct a phytochemical analysis of an invasive weed-type variety of *Cannabis sativa*, focusing on qualitative and quantitative determinations. The literature is limited regarding the phytochemical composition and potential applications of this invasive species, therefore, we aimed to assess the potential utility of this invasive plant, with an emphasis on the relevance of its practical utilization.

2. Materials and methods

Chemicals

Folin-Ciocâlteu reagent was purchased from Carl Roth GmbH (Karlsruhe, Germany), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and Trolox were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). ABTS tablets [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)], 50 mg/tablet were purchased from Roche Diagnostics GmbH (Germany). All other solvents used in this research were of analytical grade.

Plant material and extract preparation

The herbal drug (aerial parts) was collected from a sunflower (*Helianthus annuus*) culture from Bărăganului plain, Romania, in August 2022. The plant material was dried in the shade and deposited in laboratory conditions until analysis. A voucher specimen (SF-CS-22) was deposited at the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș, Romania. The sample collected presents the morphological characteristics of the *Cannabis sativa* subsp. *sativa* var. *spontanea*.

An aliquot of the herbal drug was subjected to artificial drying in an oven, at 121 °C, for an hour. Hexane and methanol extractions were performed from the artificial dried herbal drug, giving the following extracts CS-DH (hexane) and CS-DM (methanol). Also, methanolic extracts (1%) were prepared using the herbal drug dried in laboratory conditions, in an ultrasonic water bath at 40 °C, for 30 minutes giving the extract abbreviated CS-M.

Micro-anatomical characterization of the herbal drug

Leaf samples were sectioned in the elder stem, and an 80% chloral hydrate solution was used for the clarification of the cross sections. The sections were mounted in a gelatine-glycerin mixture and analyzed with a Micros-Austria microscope.

Identification of cannabinoids by thin-layer chromatography (TLC)

CS-DM, CS-DM, and CS-M extracts were applied to the TLC plates. A 0.01% CBD solution was used as standard, and aliquots from commercial CBD oils were used as control.

The solutions were applied as bands (1 cm) on silicagel 60 F_{254} plates (Merck). Plates were developed in the ascending mode in a saturated chamber using a mixture of hexane: diethyl ether (80:20, *v*/*v*) as the mobile phase. Following development, the dried plates were sprayed with a 0.5% vanillin solution of methanol-hydrochloric acid. The plates were analyzed in visible and at UV_{254} (Galand et al., 2004; Liu et al., 2020; Sherma and Rabel, 2019).

Identification of flavonoids by thin-layer chromatography (TLC)

For the identification of flavonoids, the methanolic extract was used (CS-M), as well as

0.05% methanolic solutions of chlorogenic acid, rutoside, and caffeic acid. The solutions were applied as bands (1 cm) on silicagel 60 F_{254} plates (Merck). Plates were developed in the ascending mode in a saturated chamber using a mixture of ethyl acetate: formic acid: acetic acid: water; 100:11:11:27 (*v/v/v/v*) as mobile phase. Following development, the dried plates were sprayed with Natural substance-polyethylene glycol reagent (NST/PEG). The plates were analyzed in visible and at UV_{365} (Sherma and Rabel, 2019).

Total flavonoid content (TFC)

Total flavonoid content was determined after the method described in the Romanian Pharmacopoeia 10th Edition (*Farmacopeea Română*, 1993). Briefly, 10 mL of the methanolic extract (CS-M) was diluted at 25 mL with methanol and filtered. An aliquot of this solution was mixed with 5 mL of 10% sodium acetate solution, 3 mL of 2.5% aluminum chloride solution, and methanol. The absorbance was measured at 430 nm after 15 minutes against a blank solution. The test was carried out in triplicate. A calibration curve was prepared using a standard solution of quercetin in the concentration range of 0.004 - 0.012 mg/mL.

Total flavonoid content was estimated from the calibration curve of quercetin (R^2 = 0.9948), and the results were expressed as mg quercetin equivalents per gram dry weight (mg QE/ g DW).

Total polyphenolic content (TPC)

The total polyphenolic content was determined after a modified method described in the European Pharmacopoeia 10th edition. Briefly, 40 µL from the methanolic extract (CS-M) were mixed with 3160 µL water, 200 µL Folin-Ciocâlteu reagent, and 600 µL of 9% sodium carbonate solution. The mixture was allowed to stand in the dark at room temperature for 30 minutes. The absorbance was read at 765 nm against a suitable blank. Total polyphenolic content was estimated from the calibration curve of gallic acid (0.002 - 0.025 mg/ mL, R^2 = 0.9649). The results were expressed as mg gallic acid equivalents per gram dry weight (mg GAE/ g DW).

Total hydroxycinnamic derivatives content (HDC)

HDC was determined according to the method described in the European Pharmacopoeia 10th edition (*European Pharmacopoeia (Ph. Eur.)*, 2010), using the CS-M extract. The results were expressed as mg rosmarinic acid equivalents per gram of dry weight (mg RAE/ g DW), and was calculated using equation 1, taking the specific absorbance of rosmarinic acid to be 400:

$$HDC = \frac{A \times 2.5}{m} \times 10 \quad (1)$$

where: A - absorbance of the test solution at 505 nm

m - mass of the substance to be examined, in grams

Ion chromatography analysis

Inorganic anion composition was determined using a Dionex ICS-3000 Ion Chromatography System and a Dionex IonPac® AS23 analytical column under suppressed conditions. The eluent used was sodium carbonate 4.5 mM/sodium bicarbonate 0.8 mM, its flow rate was 1.2 mL/min at 30 °C. The detection was conductometric and the analysis time was 20 minutes. Combined Seven Anion Standard II (Thermo Scientific) was used for the identification and quantification of anions in Cs-M extract.

DPPH• assay

Antioxidant activity with DPPH• was determined according to the method published by Laczko-Zöld et al (Laczkó-Zöld et al., 2018). Briefly, 150 µL of extract (CS-M) in different concentrations were mixed with 200 µL DPPH• 0.1 mM solution in a 96 multiwell plate. The mixtures were allowed to stand for 30 minutes, at room temperature, and the absorbance was determined at 517 nm using an Epoch multiplate reader (BioTek Instruments Inc., USA). The inhibition capacity was calculated using Equation 2:

$$IC\% = \frac{A_0 - A_1}{A_0} \; x \; 100 \qquad (2)$$

Where: A_0 - absorbance of DPPH• solution, A_1 - the absorbance of the sample solution

The concentration that inhibits 50% of the DPPH• activity (IC_{50}) was calculated by plotting the absorbance of the solution against the logarithm of the concentrations.

Ascorbic acid was used as positive control.

$ABTS^{+\bullet}$ radical scavenging assay

Antioxidant activity with $ABTS^{+\bullet}$ was determined according to a previously described method, with slight modifications (Ştefănescu et al., 2017). The absorbance was measured at 734 nm using an Epoch multiplate reader (BioTek Instruments Inc., USA). The inhibition capacity and IC_{50} were calculated as described in the DPPH• assay. Trolox was used as a positive control.

α-Amylase inhibition capacity

The inhibition capacity of the methanolic extract (CS-M) on α-amylase was determined according to a previously published method (Ercan and El, 2016). The DNS reagent was prepared by mixing 8 mL of 5.3 M sodium potassium tartrate tetrahydrate solution in 2 M NaOH, 20 mL of 96 mM 3, 5- dinitrosalicylic acid solution, and 12 mL of water. 0.5 mL of different concentrations of the extract was incubated for 30 minutes with the α-amylase solution (1 U/mL) before the starch solution was added. Further, the mixture was incubated at 37 °C, for 10 minutes. The reaction was stopped by adding the DNS reagent, and the samples were introduced in a boiling water bath for 10 minutes. The absorbance of the solution was measured at 540 nm. One negative control (absence of inhibitor) was set up to obtain 100% enzyme activity. Acarbose was used as a positive control. The inhibition capacity was calculated using the following formula (Equation 3):

$$IC\% = 100 \; x \; (1 - \frac{A \; sample}{A \; control}) \qquad (3)$$

The results were expressed as IC_{50} in mg/mL.

Statistical analysis

All experiments were done in triplicate (n = 3) and results were expressed as means ± standard deviation (SD). The statistical analyses were performed by using GraphPad Prism 9.0 software (San Diego, CA). Statistical differences in the antioxidant activities were determined through analysis of variance (ANOVA). Student *t*-test was used to compare the IC_{50} of the extract and acarbose. A value of *p* lower than 0.05 was considered to be statistically significant.

3. Results and discussions

Micro-anatomical characterization of the herbal drug

The analysis of the cross-section through the leaf revealed a heterogeneous-asymmetric (bifacial) structure; the upper epidermis and the

lower epidermis are made up of a single layer of cells, which delimits the mesophyll made up of palisade tissue and lacunar tissue. Numerous cystolithic trichomes are observed on the upper epidermis.

There are three types of glandular trichomes characteristic of *Cannabis sativa*: bulbous glandular trichome, capitate-stalked glandular trichome, and capitate-sessile glandular trichome. Cannabinoids are synthesized and stored in these trichomes (Raman et al., 2017).

There are also two types of non-glandular, unicellular trichomes: cystolithic trichomes (cystoliths of calcium carbonate) and long and slender trichomes (Raman et al., 2017).

The microscopic analysis of the herbal drug confirmed the identity of the species. As it can be seen in **Figure 1**, numerous trichomes can be found in the herbal drug. The glandular trichomes are responsible for the secretion of the oleoresin (Gurav and Gurav, 2014).

Identification of cannabinoids by thin-layer chromatography (TLC)

The TLC (**Fig. 2.**) analysis revealed that CBD is found in a reduced concentration in the sample dried in laboratory conditions, while the sample dried in the oven at 121 °C for 1 hour, contains higher concentrations of CBD. This conclusion could be drawn based on the correlation between the intensity and dimensions of the CBD - CBD-corresponding zone. This higher concentration of CBD was probably generated through the decarboxylation of its corresponding acid (cannabidiolic acid - CBDA) (**Fig. 3.**).

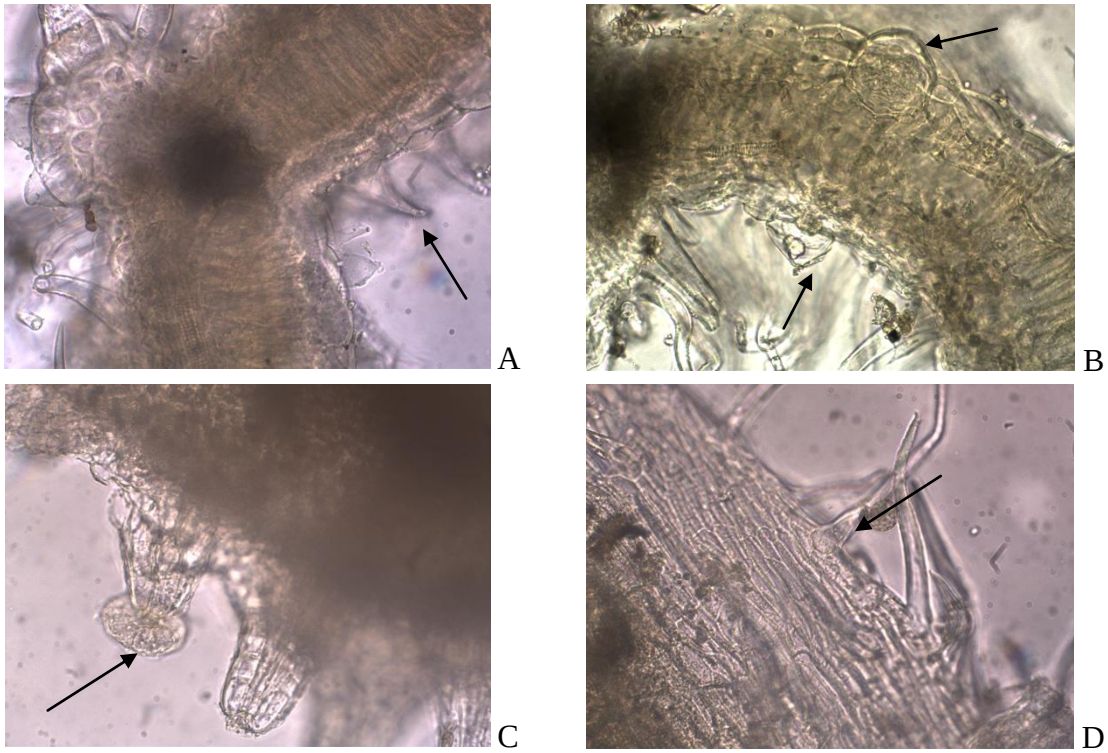

Fig. 1. Microscopic characteristics of the herbal drug: A - non-glandular cystolith-containing conical trichomes; B - Capitate-sessile glandular trichome; C- Capitate-stalked glandular trichome, D - non-glandular longer and slender trichomes

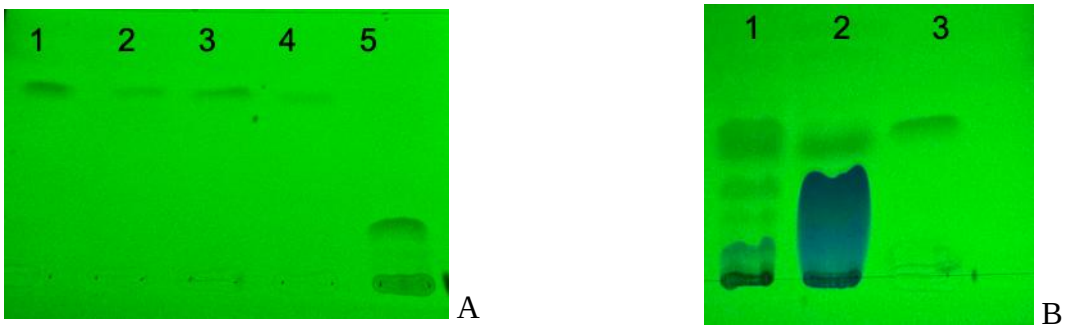

Fig. 2. A: 1 - CBD 0.01% solution, 2 - CBD oil 5%, 3 - CBD oil 10%, 4 - CBD extract 5%, 5 - CS-M; B: 1 - CS-DH, 2 - CS-DM, 3 - CBD 0.01% solution

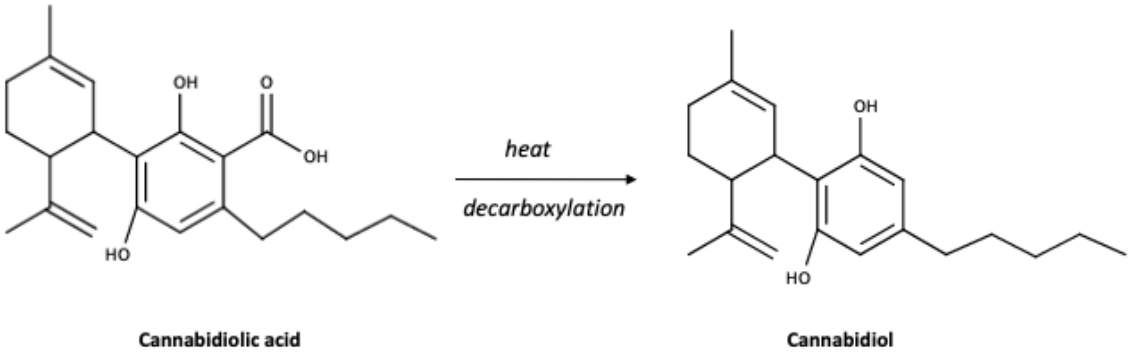

Fig. 3. Decarboxylation reaction of CBDA to CBD

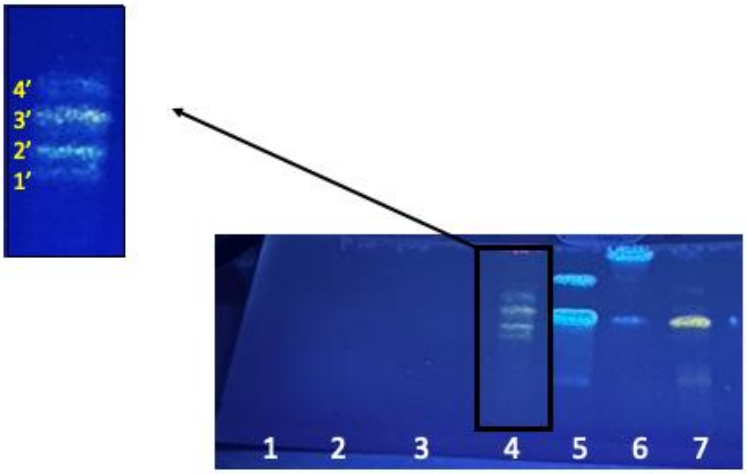

Fig. 4. TLC analysis of flavonoids from *Cannabis sativa*; 1 - 5% CBD oil, 10% CBD oil, CBD extract, 4 - sample, 5 - chlorogenic acid, 6 - caffeic acid, 7 - rutoside

Identification of flavonoids by thin-layer chromatography (TLC)

Separation of flavonoids by TLC revealed the presence of 4 compounds in the sample (**Fig. 4.**). The compounds had different retention factors compared with the standard solutions used. Based on their fluorescence in UV_{365} we can presume that the compounds could be the characteristic *Cannabis* prenilated flavones, cannflavins, but as well, these compounds could be flavons or flavonols derivatives. An HPLC analysis is needed in

order to identify the flavonoids extracted from this herbal drug.

TPC, TFC, HDC

Cannabis sativa is used for its cannabinoid content. However, only a few research papers have described the polyphenolic composition of the herbal drug.

The results obtained in this study (**Table 1.**) showed that the concentration of polyphenolic compounds in the herbal drug is low. Compared to other published data, our samples contained lower concentrations of TPC and flavonoids. However, Hacke *et al*. have used supercritical fluid extraction for extract preparation, which could have led to a better yield (Hacke et al., 2019). It is well known that geographical variability is mainly responsible for the different synthesis of secondary metabolites.

With regard to the flavonoid content, the results obtained in our study are similar to the results obtained by Hacke et al (Hacke et al., 2019).

IC analysis

Ion chromatographic analysis showed the presence of traces of fluoride, chloride, nitrate, phosphate, and sulfate ions (**Table 2.**). Four unidentified anions, possibly low molecular weight organic acids, were also present (**Fig. 5.**).

DPPH and ABTS assay

Antioxidant radical scavenging activity was measured through DPPH and ABTS assays. These methods are frequently used for screening possible antioxidant activities of natural compounds (Gulcin, 2020).

Interestingly, although the total phenolic, flavonoid, and hydroxycinnamic acid composition is quite low, compared to other herbal drugs, the methanolic extract has a powerful antioxidant activity determined with the DPPH assay (**Fig. 6.**). This suggests that other compounds from the extract are contributing to this activity. The potential of the active compounds from the extracts to scavenge the ABTS radical was lower compared with the DPPH assay. This result is probably linked with the ATBS assay being more suitable for exploring the radical scavenging activity of hydrophilic compounds, and with the lipophilicity of cannabinoids and terpenes, it was predictable that the antioxidant activity would be reduced in this assay. The results are in accordance with the results published by Zengin et al, who have tested the antioxidant activity of the essential oil distilled from the aerial parts of *Cannabis sativa*. They have reported that the essential oil had a good radical scavenging activity against the DPPH radical but was inactive against the $ABTS^{\bullet+}$ radical (Zengin et al., 2018).

This method showed that the extract has very good antioxidant activity, with IC_{50} = 0.068 ± 0.004 mg/mL, comparable to that of ascorbic acid used as a standard (IC_{50} = 0.053 ± 0.003 mg/mL).

Table 1. Total polyphenolic content, total flavonoid content, and total hydroxycinnamic derivatives content

TPC (mg GAE/ g)	**TFC (mg QE/ g)**	**HDC (mg RAE/ g)**
13.6 ± 1.3	7.25 ± 0.8	3.1 ± 0.3

* results are expressed as mean ± standard deviation (n = 3)

Table 2. Anion composition in the extract

No.	Retention time (min)	Name	Concentration ppm
1	3.33	Fluoride	1.895
2	3.52	n.a.	n.a.
3	3.68	n.a.	n.a.
4	4.21	n.a.	n.a.
5	5.55	Chloride	11.13
-	n.d.	Nitrite	n.d.
-	n.d.	Bromide	n.d.
6	10.12	Nitrate	3.919
7	12.95	Phosphate	4.788
8	15.00	Sulfate	2.087
9	17.30	n.a.	n.a.

n.a. - not analyzed, n.d. - not detected

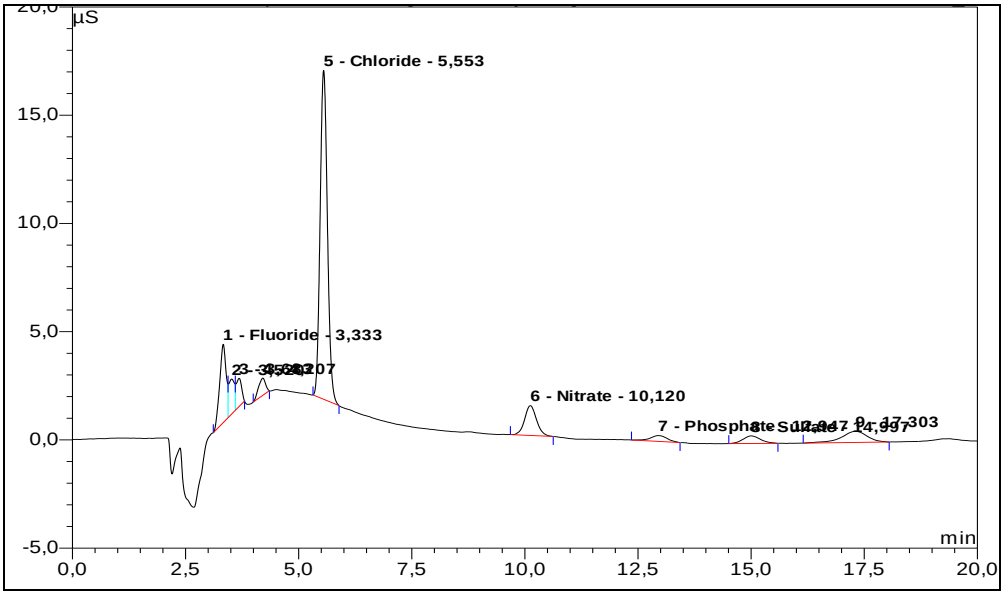

Fig. 5. IC analysis of *Cannabis sativa* extract

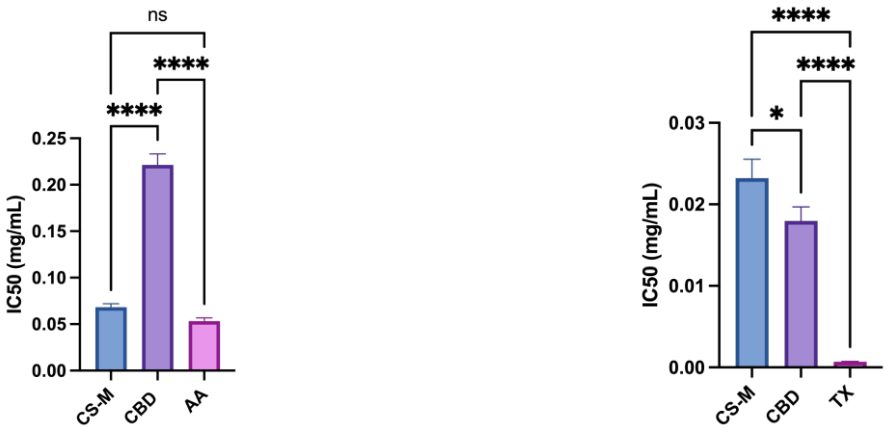

Fig. 6. DPPH and ABTS results; CS - M - *Cannabis sativa* methanolic extract, CBD - cannabidiol, AA - Ascorbic acid, TX - Trolox; *statistically significant difference at $p < 0.05$; ****statistically significant difference at $p < 0.0001$; ns - no significant difference at $p < 0.05$

α-Amylase inhibition capacity

α-Amylase is an enzyme that catalyzes the hydrolysis of starch to maltose and is used as a target for delaying postprandial hyperglycemia in diabetic patients. Usually, the action of these enzyme inhibitors is dose-dependent, and the treatment can reduce the glycated hemoglobin (HbA1c) (Agarwal amd Gupta, 2016; Rosak and Mertes, 2012). An impressive number of natural compounds were found to have the ability to inhibit one of the two enzymes involved in carbohydrate metabolism (Sales et al., 2012). There are only a few studies that have evaluated the *α*-amylase inhibitory capacity of *C. sativa* extracts, and the results are inconclusive (Hourfane et al., 2023; Shah et al., 2020).

For this assay, the concentration that inhibits 50% of the enzyme's activity (IC_{50}) was calculated from the dose-response curve by plotting the IC% against the logarithm of the concentration. The extract had an IC_{50} of 1.058 ± 0.072 mg/mL, while acarbose had a lower statistically significant IC_{50} of 0.063 ± 0.002 mg/mL at $p < 0.05$. The herbal drug proved to have a minor-moderate effect on *α*-amylase which excludes a possible application in the prevention of carbohydrate absorption.

Conclusions

In conclusion, the results of the present investigation infer that the *Cannabis sativa* extract possesses potent antioxidant activity, although the concentration of polyphenols, flavonoids, and cinnamic acids is low. Although our obtained results indicate the possibility of utilization of this product as a source of natural antioxidants, further studies are needed to evaluate the safety of this herbal drug.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

This work was supported by the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș, Research Grant number 163 /7/ 10.01.2023.

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