Journal of Pharmaceutical and Biomedical Analysis 236 (2023) 115723

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Identification of phenolic compounds from inflorescences of non-psychoactive *Cannabis sativa* L. by UHPLC-HRMS and *in vitro* assessment of the antiproliferative activity against colorectal cancer

Clarissa Caroli [a,b], Virginia Brighenti [a], Alice Cattivelli [c], Stefano Salamone [d], Federica Pollastro [d], Davide Tagliazucchi [c,*], Federica Pellati [a,*]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, Via Giuseppe Campi 103, 41125 Modena, Italy
[b] Clinical and Experimental Medicine PhD Program, University of Modena and Reggio, Via Giuseppe Campi 287, 41125 Modena, Italy
[c] Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola 2, 42100, Reggio Emilia, Italy
[d] Department of Pharmaceutical Sciences, University of Eastern Piedmont, Largo Donegani 2, 28100 Novara, Italy

ARTICLE INFO

Keywords:
Cannabis sativa L.
Polyphenols
Extraction
UHPLC
HRMS
Antiproliferative activity

ABSTRACT

Phenolic compounds from *Cannabis sativa* L. (Cannabaceae family), in particular cannflavins, are known to possess several biological properties. However, their antiproliferative activity, being of great interest from a medicinal chemistry point of view, has not been deeply investigated so far in the literature. In the light of this, the aim of this study was to obtain an enriched fraction of polyphenols (namely PEF) from inflorescences of a non-psychoactive *C. sativa* (hemp) variety and to evaluate its antiproliferative activity against cancer cells, capitalizing on a new and selective extraction method for hemp polyphenols, followed by preparative flash column chromatography. Untargeted metabolomics, using a new method based on ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS), was applied here for the first time to fully characterize PEF. Then, the main phenolic compounds were quantified by HPLC-UV. The antiproliferative activity of PEF and of the isolated compounds was assessed *in vitro* for the first time against Caco-2 and SW480 human colon adenocarcinoma cell lines providing promising IC_{50} values, in comparison with the reference drug used in therapy for this cancer type. Based on these results, PEF can be considered as a new highly potential therapeutic product to be further investigated against colorectal cancer, thanks to the possible synergistic interaction of its compounds.

1. Introduction

Colorectal cancer (CRC) is one of the most diagnosed common cancers in high-income countries (HIC) [1], representing the 10% of diagnosed tumors in 2020, according to the World Health Organization (WHO) [2]. The causes of this trend can be mainly attributed to dietary and lifestyle changes in HIC, leading to an increase of overweight, which is one of the main risk factors associated to CRC [1,3]. In recent years, the morbidity and mortality rates due to this disease are increasing, representing the second cause of decease in cancer-related death, with a high incidence of metastasis [4]. Another main concern in CRC is that it can easily develop multidrug resistance (MDR), with consequent reduction or inefficacy of current anticancer drugs [5].

For these reasons, it is crucial to improve current therapeutic treatments by investigating new possible active compounds that can overcome this problem. Polyphenols are a large class of natural compounds, some of which can act against cancer cells with several mechanisms of action, thus becoming highly interesting compounds to be exploited in this field [6]. As an example, quercetin has already demonstrated to possess antiproliferative activity against colon cancer cell lines by modulating the expression of cannabinoid receptor 1 (CB1) [7]. In addition, other flavonoids, such as apigenin and luteolin, inhibit the proliferation of colon cancer cell lines [8]. One of the mechanisms of action exerted by apigenin is the prevention of the migration of pyruvate kinase M2, while luteolin can promote apoptosis by reducing the overexpression of antiapoptotic proteins [8]. Moreover, it has been demonstrated that both quercetin and luteolin can enhance the effect of the anticancer drug 5-fluorouracil (5-FU), thus reducing the toxic effects

* Corresponding authors.
E-mail addresses: davide.tagliazucchi@unimore.it (D. Tagliazucchi), federica.pellati@unimore.it (F. Pellati).

https://doi.org/10.1016/j.jpba.2023.115723
Received 3 August 2023; Received in revised form 4 September 2023; Accepted 12 September 2023
Available online 14 September 2023

related to its use in CRC [7].

Polyphenols are widely present in nature and they have also been detected in *Cannabis sativa* L., a well-known plant belonging to the *Cannabaceae* family [9,10], which includes both psychoactive and non-psychoactive varieties, based on the content of Δ^9-tetrahydrocannabinol (Δ^9-THC) [11]. In addition to cannabinoids, *C. sativa* is a source of several bioactive compounds, belonging to different chemical classes, including terpenes and polyphenols [10]. In particular, *C. sativa* contains a peculiar class of compounds belonging to prenylated flavones, namely cannflavins [10,12]. These molecules have already demonstrated to possess antioxidant, anti-inflammatory, neuroprotective and antiparasitic activities [12,13]. However, there are few data on their antiproliferative activity [13,14], which has been more frequently assessed for cannabinoids, with particular regard to cannabidiol (CBD) [15]. Fig. 1 shows the chemical structures of cannflavin A (CFL-A) and B (CFL-B), and of their demethoxy derivatives, i.e. demethoxy cannflavin A (demethoxy CFL-A) and demethoxy cannflavin B (demethoxy CFL-B) [13,16].

High-performance liquid chromatography (HPLC), coupled with diverse types of detectors, is the analytical technique of choice for the identification of phenolic compounds in complex mixtures, such as plant extracts [9,16]. In particular, ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS) and HPLC-UV are applied to obtain reliable qualitative and quantitative data on the numerous compounds that compose a natural extract [9,10,16].

In the light of all the above, the aim of this work was to obtain a polyphenol-enriched fraction (PEF) from decarboxylated hemp inflorescences by developing efficient extraction and purification procedures. UHPLC-HRMS was extensively applied for the first time to perform an untargeted metabolomics of polyphenols in PEF and, together with the quantification by HPLC-UV, to guide the isolation of its main phenolic constituents. Finally, both PEF and pure compounds were tested *in vitro* for the first time to evaluate their antiproliferative activity against human colon adenocarcinoma cell lines, in comparison with a conventional anticancer drug currently used in chemotherapy.

2. Material and Methods

2.1. Chemicals and solvents

Analytical grade petroleum ether (PE), diethyl ether, *n*-hexane, ethyl acetate (EtOAc) and HPLC grade acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), acetone, formic acid (HCOOH), sulfuric acid (H_2SO_4), gallic acid, the Fast Blue B reagent and cisplatin were purchased from Sigma-Aldrich (Milan, Italy). Pure *N-trans*-feruloyltyramine (purity ≥ 98%) was purchased from Vinci Biochem s.r.l. (Florence, Italy). Reference solution of CBD and cannabidiolic acid (CBDA) at 1.0 mg/mL in MeOH and ACN, respectively, were purchased from Restek (Milan, Italy). Water (H_2O) was purified by using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

Silica gel 60 (particle size 0.063–0.200 mm), reversed-phase (RP) C_{18} silica gel (particle size 25 μm) and Celite® 545 (particle size 0.02–0.1 mm), used for low-pressure chromatography (LPC), were purchased from Macherey-Nagel (Düren, Germany).

2.2. Extraction of hemp inflorescences

Hemp plant material (inflorescences), belonging to the Kompolti variety, was kindly provided by Materia Medica Processing s.r.l. (Siena, Italy). This variety is approved for commercial use by the European Union (EU) and it is certified for a content of Δ^9 -THC below 0.3% (w/w) [17].

Fifty grams of grounded hemp inflorescences were placed in an oven for 15 min at a temperature of 110 °C to remove volatile compounds belonging to the class of terpenes. The temperature was then raised at 120 °C and it was kept constant for 60 min to achieve a complete decarboxylation of cannabinoic acids into their neutral cannabinoids. The decarboxylated plant material was then submitted to a dynamic maceration with 300 mL of *n*-hexane at room temperature for 15 min to remove lipophilic compounds. The extract was then paper filtered, with the filtrate sent to waste. This procedure was repeated other three times with additional 200 mL of *n*-hexane. The residual plant material was then submitted to a dynamic maceration with 300 mL of a MeOH/acetone mixture (90:10 v/v) with 0.1% HCOOH at room temperature for 15 min. The extract was then paper filtered, with the solid residue extracted with the same procedure other three times with additional 200 mL of the extraction solvent. The extracts were combined, and they were brought to dryness under vacuum with a rotary evaporator at 30 °C. The raw extract yield was 4.0% (w/w).

2.3. Purification of PEF by preparative flash column chromatography

The crude extract (0.5 g) was further purified to remove cannabinoids and other co-extracted compounds using an Isolera™ One automated flash purification system with UV detection (Biotage AB, Uppsala, Sweden). Separation was achieved on a silica gel SNAP cartridge (50 g) (Biotage AB, Uppala, Sweden), with a mobile phase composed of PE (A)

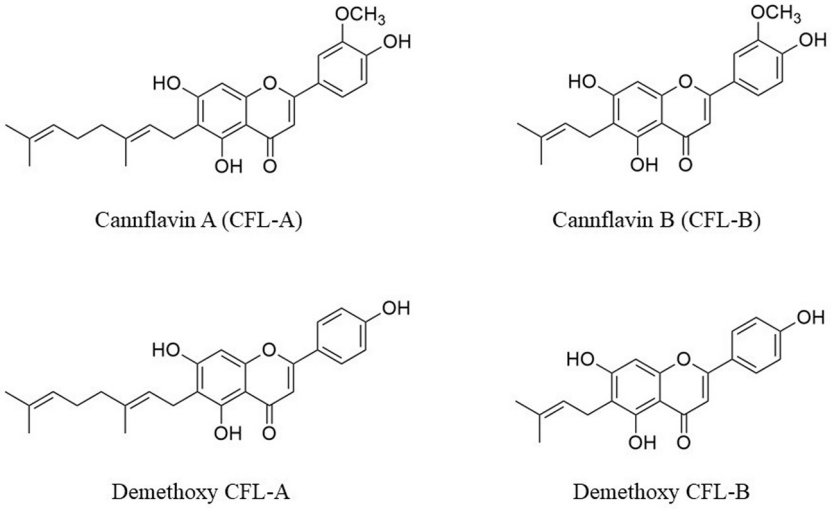

Fig. 1. Chemical structures of cannflavins from hemp.

and acetone (B) at the flow rate of 50 mL/min. The gradient elution was set as follows: 0–15 min from 7% to 60% B. The volume of the collected fractions was 18 mL.

The purification of PEF was monitored by TLC on Merck silica gel 60 F254 (0.25 mm) plates and visualized by staining with 5% H_2SO_4 in EtOH and heating. The fractions collected were also analyzed by TLC together with standards of both CBDA and CBD, to easily identify and remove those containing cannabinoids, using a solution containing the Fast Blue B reagent. Cannabinoid-free fractions were combined, and they were brought to dryness under vacuum with a rotary evaporator at 30 °C to obtain PEF (98.0 mg, yield 18.8%, w/w).

2.4. Sample preparation for HPLC analysis of PEF

For the HPLC analysis, approximately 5 mg of PEF were dissolved in 5 mL of the extraction solvent and filtered through a 0.45 μm PTFE filter prior to the injection into the HPLC system. The sample preparation was performed in duplicate, and four injections were performed for each solution.

2.5. UHPLC-HRMS analysis of PEF

The qualitative analysis of PEF was performed by UHPLC-HRMS. The analyses were carried out on a Thermo Scientific (Waltham, MA, USA) UHPLC Ultimate 3000, equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electro-spray ionization (HESI) source.

Separation of the analytes was achieved on an Ascentis Express C_{18} column (150 mm × 3.0 mm I.D., 2.7 μm, Supelco, Bellefonte, PA, USA), with a mobile phase composed of 0.1% HCOOH in both H_2O (A) and ACN (B). The gradient elution was modified as follows: 0–20 min from 2 to 25% B, 20–30 min from 25 to 40% B, 30–40 min from 40 to 80% B which was kept for 5 min, 45–55 min from 80 to 90% B which was kept for 5 min. The post-running time was 10 min. The flow-rate was 0.3 mL/min. The column temperature was set at 30 °C. The sample injection volume was 4 μL.

MS acquisition was carried out with a heated electro-spray ionization source operated in both the positive and in the negative ion mode. As to the MS detector, the source parameters were set as follows: sheath gas (N_2) 40, auxiliary gas (N_2) 30, auxiliary gas temperature 290 °C, electrospray voltage 3.5 kV (+) and 3.2 kV (−). The analyses were acquired at a resolving power of 70.000 full width at half maximum (FWHM). The other mass analyzer parameters were set as follows: scan range *m/z* 100–1000, automatic grain control (AGC) target 1×10^6 ions in the Orbitrap analyzer, ion injection time 243 ms and isolation window for the filtration of the precursor ions *m/z* 3.0. The fragmentation of precursors ions was performed at 20, 30 and 50 as normalized collision energies (NCE).

2.6. HPLC-UV analysis of PEF

The quantitative analysis of PEF was performed by HPLC-UV. Analyses were carried out on a Shimadzu (Kyoto, Japan) Prominence UFLC XR System, equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Shimadzu SPD-10A VP HPLC System UV–VIS Detector. The HPLC column and the applied chromatographic conditions were the same as those reported for the UHPLC-HRMS system. UV/Vis spectra were set in the range 190–600 nm. Chromatograms were acquired at 210 and 342 nm.

2.7. Extraction and isolation of cannflavins

The plant material (500 g, inflorescences and leaves from the Carmagnola variety, Canvasalus s.r.l., Monselice, Italy) [17] was decarboxylated, as previously described for the preparation of PEF, and extracted with acetone (10 L × 2) in a vertical percolator at room temperature, affording 30.3 g (6.1%) of a dark green syrup. In order to remove saturated fatty acids and waxes with cold condensation, this was later dissolved at 45 °C in 300 mL of MeOH (raw extract-MeOH ratio 1:10, w/v) and left at–10 °C overnight. After that, the solution was vacuum-filtered with cold MeOH in a sintered funnel protected by a bed of stratified Celite®, MeOH evaporated in a rotary evaporator to obtain 22.5 g of residual extract. This latter part was purified by solid-phase extraction on C_{18} silica gel to remove chlorophylls and the unsaturated fatty acids. For this purpose, the extract was dissolved in the minimal MeOH amount at 45 °C and charged on 66 g of C_{18} (extract-stationary phase ratio 1:3, w/w), packed with MeOH in a sintered funnel with a side arm for vacuum. Elution with MeOH provided 19.3 g of fraction. This latter fraction was purified by LPC on silica gel (500 g, PE-EtOAc gradient from 90:10–20:80, v/v) to afford three fractions (I, II, and III). Fraction III (4.7 g) was further purified using the Isolera™ One automated system with a SNAP ULTRA C_{18} (12 g) (solvent A: MeOH + 0.3% HCOOH, solvent B: H_2O + 0.3%, gradient from 50:50–95:5 v/v) to afford, after crystallization with diethyl ether, 43.5 mg of CFL-A (yield 0.009%) and 19.0 mg of CFL-B (yield 0.004%) both as a yellow powder [18]. The purity of the isolated compounds was 99% for CFL-A and 97% for CFL-B, respectively, as determined by HPLC [10]. All the isolated compounds were identified according to ^{1}H NMR previously described in the literature [18]. ^{1}H 400 MHz NMR spectra were recorded on a Bruker 400 spectrometers (Bruker, Billerica, MA, USA). Chemical shifts were referenced to the residual solvent signal (C_3D_6O: $\delta_H = 2.05$). ^{1}H NMR data of the isolated compounds CFL-A and CFL-B are shown in Figs. S1-S2 of the Supporting Information, respectively.

2.8. Standard solutions for HPLC quantitative analysis

The stock standard solution of each compound (CFL-A, CFL-B and *N-trans*-feruloyltyramine) was prepared as follows: an accurate weight of pure compound was measured (0.20, 0.17 and 0.16 mg, respectively) and transferred into a 1 mL volumetric flask. The compounds were then dissolved and diluted to volume with the extraction solvent and the external standard calibration curve was generated by using six data points. Four μL of each reference solution were used for the HPLC analysis and the injections were performed in triplicate for each concentration level. Calibration curves for CFL-A, CFL-B and *N-trans*-feruloyltyramine were constructed at six calibration levels in the range 2.0–100.0, 1.7–85.0 and 4.0–80.0 μg/mL, respectively, by plotting the peak areas of the analytes *vs*. their concentration. The correlation coefficient r^2 was higher than 0.998. The amount of demethoxy CFL-A and of demethoxy CFL-B was determined by using the calibration curve of CFL-A and CFL-B.

2.9. Determination of the in vitro antiproliferative activity of PEF and pure compounds

Human colon adenocarcinoma cell lines Caco-2 and SW480 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Leibowitz medium (L-15), respectively. For cell growth, both the cell media were supplemented with 10% fetal bovine serum, 1% antibiotic mix (streptomycin and penicillin), and 2 mM L glutamine. Cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2.

PEF (50 mg) was dissolved in 1 mL of dimethyl-sulfoxide (DMSO). Pure phenolic compounds (CFL-A, CFL-B and *N-trans*-feruloyltyramine) and cisplatin were dissolved in DMSO at a concentration of 50 mM. For the antiproliferative assay, PEF, hemp compounds and cisplatin solutions were diluted at the desired concentrations in the respective cell culture media and filtered at 0.2 μm. The final concentration of DMSO was ≤ 0.5%.

Before the treatments, cells were seeded in a 96-well plate at a density of 4×10^3 and 8×10^3 for Caco-2 and SW480, respectively, and

left to adhere for 24 h before the addition of 200 μL of the selected compounds at different concentrations. After 24 and 48 h of incubation, the antiproliferative activity was assessed as previously described using the MTS assay [19]. Control experiments (100% of proliferation) were carried out by incubating cells with the respective cell media containing 0.5% DMSO.

Results were expressed as IC_{50} values, defined as the compound concentration able to inhibit cell growth by 50%, calculated by non-linear regression analysis by plotting the base-10 logarithm of the compound concentration as a function of the percentage of inhibition. The IC_{50} values were expressed as μg of total phenolic compounds/mL for PEF and as μM for pure compounds, respectively. Total phenolic compounds were determined in PEF following the Folin-Ciocalteu method with gallic acid (GA) as the reference standard. Total phenolic content in the re-suspended PEF was 3302 ± 264 μg of gallic acid equivalent (GAE)/mL.

3. Results and discussion

3.1. Extraction and purification of PEF

To obtain PEF from hemp inflorescences, it was necessary to optimize the extraction and purification conditions to get a final extract having polyphenols as the representative compounds. Several experiments were carried out to find the best solvent to extract as many phenolic compounds as possible. At the beginning, acetone was used as the extraction solvent [10], followed by MeOH/acetone (50:50, v/v) with 0.1% HCOOH and, finally, by MeOH/acetone (90:10 v/v) with 0.1% HCOOH. The last one allowed us to extract more polyphenols, compared to the other solvents (data non shown), and, therefore, it was chosen to be used in this study, after the removal of lipophilic compounds from the plant material using *n*-hexane. A preliminary heating step of hemp inflorescences was necessary to favor the removal of terpenes and to convert cannabinoic acids into their neutral counterparts, thus favoring their subsequent washing out with *n*-hexane. The extract was then fractionated using preparative flash column chromatography under normal phase conditions. The fractions obtained were firstly screened for cannabinoids by thin-layer chromatography (TLC) analysis and all cannabinoid-free fractions were then combined to form PEF.

3.2. Chemical characterization of PEF using UHPLC-HRMS

An untargeted metabolomic analysis was performed on PEF to provide a detailed dereplication by UHPLC-HRMS. The identification of compounds was achieved by combining both experimental MS and MS/MS data with those described in the literature [10,16,20–33]. Even if different papers show the composition of specific classes of polyphenols in hemp using chromatographic methods [10,16,20,21,24,25,34], to the best of our knowledge a comprehensive multi-component analysis of these compounds using untargeted metabolomics has never been described up to now in the literature. UHPLC conditions, including the mobile phase composition, the flow-rate and the column temperature, were optimized to achieve a good separation of phenolics. HRMS parameters were carefully set up to obtain product ions of adequate intensity. Table 1 shows the retention times, UV, MS and MS/MS data of phenolic compounds identified in PEF.

For the identification of phenolic acids, given the presence of a carboxylic group, they ionized better in the negative ion mode. Therefore, only the fragmentation observed in this acquisition mode will be further considered for their identification.

Compound **1** ($[M-H]^-$ = 137.0231) was identified as hydroxybenzoic acid, since it presents the typical main fragment at *m/z* 93.0331, generated by the loss of a carboxylic group (−44 Da) [20].

Compound **4** ($[M-H]^-$ = 163.0389) and compound **5** ($[M-H]^-$ = 195.1017) showed the loss of a carboxylic group, with the generation of a main product ion at *m/z* 119.0489 and *m/z* 151.1117, respectively. By comparing their fragmentation patterns with those described in the literature, they were identified as coumaric acid and dihydroferulic acid, respectively [21,22].

Compound **6** ($[M-H]^-$ = 187.0965) was identified as hydroxygallic acid, since it showed a main product ion at *m/z* 125.0959, generated by the loss of a carboxylic group and the subsequent loss of H_2O [23].

As for hydroxycinnamic acid amides, they ionized well both in the positive and in the negative ion modes. This class of compounds has already been described for hemp seeds [24,25], but, to the best of our knowledge, it has not been studied in detail in hemp inflorescences.

Compound **2** ($[M-H]^-$ = 298.1080) was identified as *N*-*p*-coumaroyloctopamine, since it showed a product ion at *m/z* 145.0283, that represents the coumaroyl moiety, and its fragmentation pattern was consistent with that already described in literature [24].

Compound **3** ($[M-H]^-$ = 328.1187) was identified as *N*-feruloyloctopamine, given the product ion at *m/z* 133.0521, representing the 4-hydroxy-3-methoxyphenyl moiety [26]. Moreover, the fragmentation pattern was compliant with that already described in the literature [26].

Compounds **7** and **10** ($[M+H]^+$ = 284.1281 and $[M-H]^-$ = 282.1132, respectively) generated a main product ion at *m/z* 147.0440 in the positive ion mode, attributable to the loss of the tyramine moiety [25]. In the negative ion mode, the main fragment was at *m/z* 119.0489, resulting from the loss of a tyramine moiety as well. Moreover, the *m/z* 145.0282 represents the coumaroyl moiety [24,25]. For these reasons they were identified as *N*-coumaroyltyramine isomers [25].

Compounds **8** and **11** ($[M+H]^+$ = 314.1385 and $[M-H]^-$ = 312.1237, respectively) were identified as *N*-*cis*-feruloyltyramine and *N*-*trans*-feruloyltyramine, respectively, since they generated a product ion at *m/z* 177.0546 in the positive ion mode, due to the loss of the tyramine moiety [25]. In the negative ion mode, they produced a main fragment at *m/z* 148.0518, caused by the loss of the feruloyl moiety after the CO-Cα' cleavage [24,25].

Compound **9** ($[M+H]^+$ = 344.1492, $[M-H]^-$ = 342.1342) was identified as *N*-feruloyl-3-*O*-methyldopamine. Indeed, it showed a main product ion at *m/z* 177.0546 in the positive ion mode, generated by the loss of the methyldopamine moiety [27]. In the negative ion mode, it generated the same fragment as compounds **7** and **10** at *m/z* 148.0518, corresponding to the loss of the feruloyl moiety after the CO-Cα' cleavage [24,25].

Compound **12** ($[M+H]^+$ = 328.1187, $[M-H]^-$ = 326.1036) showed the same typical fragmentation pattern of the previously described feruloyl derivatives, both in the positive and in the negative ion mode. For this reason, it was identified as terrestriamide, i.e. a *N*- feruloyl amide derivative, according to the fragmentation pattern which was compliant with that previously described in the literature for this compound [28].

For what concerns flavonoids, they ionized better in the positive ion mode and, therefore, their identification was performed in this way.

Compounds **18** ($[M+H]^+$ = 453.1909), **19** ($[M+H]^+$ = 401.1232), **21** ($[M+H]^+$ = 369.1333), **23–26** ($[M+H]^+$ = 435.1804, 453.1909, 469.1857, 469.1858 respectively), **28–30** ($[M+H]^+$ = 369.1332, 451.1751 and 437.196, respectively) and **32** ($[M+H]^+$ = 437.1958) showed a main product ion at *m/z* 313.0704, generated by the loss of a prenyl moiety. This fragmentation pattern has already been described in the literature for the chemical class of cannflavin [10,16]. In particular, compounds **18, 19**, **23–26** and **29** were identified as cannflavin derivatives, given their fragmentation pattern compliant with the structure of these compounds [10,16].

Compound **21** was tentatively identified as isocannflavin B, while compound **28** was identified as CFL-B, which was confirmed by the analysis of the isolated compound [10,16].

Compound **27** ($[M+H]^+$ = 339.1277) showed a main product ion at *m/z* 283.0598. The precursor ion of this compound differed from that of CFL-B for 30 Da, corresponding to a methoxy-group. Moreover, the fragmentation pattern resembles those of cannflavins, with the main product ion generated by the loss of the prenyl moiety. For this reason, it

Table 1
Retention time, UV, MS, and MS/MS data of the compounds identified in PEF using UHPLC-HRMS.

Peak number	Compound	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$	MS/MS	$[M-H]^-$	MS/MS	Ref
1	Hydroxybenzoic acid	11.3	214, 232, 256	139.0390	140.1433 (77), 121.0286 (45), 100.1125 (14), 95.0496 (100)	137.0231	93.0331 (100)	[20]
2	*N-p*-Coumaroyloctopamine	16.8	240, 302	-	-	298.1080	280.0977 (74), 145.0283 (100), 134.0599 (16), 133.0520 (14), 119.0489 (91), 117.0332 (52)	[24]
3	*N*-Feruloyloctopamine	17.9	238, 304	-	-	328.1187	310.1088 (65), 295.0852 (16), 164.8353 (20), 161.0233 (100), 133.0521 (83)	[26]
4	Coumaric acid	18.0	232, 300, 310	-	-	163.0389	162.8382 (12), 119.0489 (100)	[21]
5	Dihydroferulic acid	19.3	248, 282	197.1172	179.1066 (100), 161.0961 (28), 135.1168 (54), 133.1012 (46), 107.0859 (47)	195.1017	160.8409 (34), 151.1117 (100), 135.0803 (73), 95.0488 (80)	[22]
6	Hydroxygallic acid	21.7	244, 296	-	-	187.0965	125.0959 (100), 97.0644 (14)	[23]
7	*N*-Coumaroyltyramine	22.5	222, 290, 310	284.1281	147.0440 (100), 121.0650 (39), 119.0493 (11)	282.1132	162.0549 (11), 145.0282 (7), 132.0568 (8), 119.0489 (100)	[24, 25]
8	*N-cis*-Feruloyltyramine	23.3	220, 288, 320	314.1385	177.0546 (100), 145.0284 (33), 121.0650 (40), 117.0337 (7)	312.1237	312.1239 (60), 297.1003 (19), 190.0500 (26), 178.0499 (47), 148.0518 (100), 135.0439 (35)	[24, 25]
9	*N*-Feruloyl-3-*O*-methyldopamine	23.9	244, 282	344.1492	177.0546 (100), 145.0284 (39), 117.0338 (9)	342.1342	342.1344 (74), 327.1114 (35), 190.0502 (34), 178.0499 (69), 148.0518 (100), 135.0439 (58)	[27]
10	*N*-Coumaroyltyramine	24.2	222, 290, 310	284.1281	284.1275 (13), 147.0440 (100), 121.0650 (39), 119.0493 (11)	282.1132	282.1133 (50), 162.0549 (13), 145.0282 (7), 136.0756 (6), 132.0566 (7), 119.0489 (100)	[24, 25]
11	*N-trans*-Feruloyltyramine	24.9	220, 288, 320	314.1385	314.1385 (19), 177.0546 (100), 145.0284 (32), 121.0650 (42), 117.0337 (7)	312.1237	312.1241 (46), 297.1005 (21), 190.0500 (30), 178.0500 (50), 148.0518 (100), 135.0439 (34)	[24, 25]
12	Terrestriamide	26.1	244, 288, 322	328.1187	328.1540 (45), 177.0546 (100), 145.0284 (35), 121.0650 (51)	326.1036	326.1396 (75), 204.0658 (49), 192.0657 (36), 148.0518 (100), 135.0439 (81)	[28]
13	Deoxyrhapontigenin (4′-*O*-Methylresveratrol)	26.8	220, 284, 320	243.1017	245.1171 (100), 243.1015 (27), 213.0908 (16), 185.0961 (7), 177.0546 (12)	241.0867	226.0631 (58), 241.0869 (80), 210.0679 (100), 197.0595 (27)	[30]
14	Apigenin	29.5	218, 268, 334	271.0601	271.0598 (100), 153.0182 (13), 147.0441 (4)	269.0452	269.0456 (100), 151.0029 (18), 149.0234 (15), 117.0332 (67), 107.0124 (12), 65.0016 (12)	[16, 31]
15	Diosmetin/chrysoeriol	30.1	222, 350	301.0706	301.0707 (100), 286.0471 (37), 258.0522 (14)	299.0557	284.0322 (100), 299.0556 (31), 256.0363 (32), 227.0346 (7), 107.0120 (9), 63.0225 (6)	[16]
16	Diosmetin/chrysoeriol	30.3	222, 350	301.0707	301.0706 (100), 286.0470 (37), 258.0522 (14)	299.0558	284.0324 (100), 256.0378 (6), 299.0558 (31), 107.0125 (7), 83.0124 (5), 63.0225 (8)	[16]
17	Cannabisin F	32.5	220, 280, 320	625.2544	462.1908 (24), 351.0859 (71), 325.1068 (100), 307.0963 (31), 121.0649 (37)	-	-	[25]
18	Cannflavin derivative	34.5	216, 274, 342	453.1909	435.1801 (5), 313.0704 (100), 298.0471 (10), 295.0599 (11)	-	-	*
19	Cannflavin derivative	34.7	214, 274, 342	401.1232	325.0704 (100), 367.1175 (52), 313.0705 (96), 310.0471 (39), 297.0757 (44)	-	-	*
20	Acacetin	35.9	244, 274	285.0757	285.0757 (100), 285.1846 (6), 270.0521 (12), 242.1572 (14)	283.0608	239.0423 (63), 211.0394 (87), 107.0124 (87), 63.0226 (100)	[16]
21	Isocannflavin B	37.2	222, 276, 344	369.1333	369.1331 (88), 313.0704 (100), 298.0470 (24)	367.1185	367.1186 (100), 352.0951 (57), 309.0404 (69), 297.0403 (59), 269.0455 (19) 133.0282 (58)	[16]
22	Hydroxymatairesinol/ nortrachelogenin	37.3	222, 276, 344	375.2166	357.2058 (100), 339.1953 (86), 275.1276 (34), 219.1014 (33)	373.2015	355.1911 (76), 311.2012 (35), 287.1288 (23), 173.0960 (100), 158.0727 (33)	[33]
23	Cannflavin derivative	37.6	220, 272, 334	435.1804	313.0704 (100), 298.0471 (13), 183.0288 (4), 165.0182 (5)	-	-	*
24	Cannflavin derivative	38.0	216, 274, 334	453.1909	435.1804 (4), 314.0737 (22), 313.0704 (100), 298.0470 (11), 165.0182 (5)	451.1761	351.0871 (46), 309.0405 (100), 297.0401 (66), 163.0022 (16), 133.0282 (85)	*
25	Cannflavin derivative	38.4	214, 274, 342	469.1857	325.0709 (6), 313.0704 (100), 298.0471 (14), 165.0184 (6), 100.1125 (16)	467.1707	435.1458 (100), 391.1180 (31), 311.0556 (23), 297.0404 (72), 163.0027 (20), 133.0281 (47)	*

(*continued on next page*)

Table 1 (*continued*)

Peak number	Compound	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$	MS/MS	$[M-H]^-$	MS/MS	Ref
26	Cannflavin derivative	38.6	216, 274, 342	469.1858	313.0704 (100), 298.0469 (14), 183.0290 (4), 165.0181 (5), 100.1125 (55)	467.1706	449.1614 (43), 351.0874 (48), 344.0534 (42), 309.0404 (71), 297.0399 (93), 133.0282 (100)	*
27	Demethoxy CFL-B	38.7	218, 272, 330	339.1277	339.1225 (5), 283.0598 (100), 183.0287 (4), 165.0181 (11)	337.1078	337.1079 (100), 293.0453 (15), 281.0452 (10), 161.0233 (8), 133.0645 (7), 117.0332 (63)	*
28	CFL-B	38.9	218, 274, 342	369.1332	313.0704 (100), 298.1469 (15), 165.0182 (9)	367.1182	352.0949 (49), 309.0403 (100), 297.0400 (31), 269.0453 (16), 133.0282 (61)	[10, 16]
29	Cannflavin derivative	39.3	220, 272, 342	451.1751	325.0706 (5), 313.0704 (100), 298.0470 (13), 283.0599 (6)	449.1604	351.0873 (68), 309.0403 (73), 297.0408 (33), 133.0282 (100), 83.0487 (23)	*
30	CFL-C	41.2	224, 274, 342	437.196	313.0704 (100), 298.0470 (17)	435.181	420.1578 (60), 351.0871 (93), 297.0400 (88), 268.0371 (37), 133.0282 (100)	*
31	Demethoxy CFL-A	42.8	222, 276, 332	407.1854	283.0598 (100), 183.0287 (5), 165.0181 (9)	405.1706	293.0454 (40), 281.0453 (100), 163.0026 (34), 161.0233 (52), 117.0332 (52)	[29]
32	CFL-A	43.0	220, 276, 340	437.1958	313.0704 (100), 298.0469 (14), 165.0182 (7)	435.1808	420.1585 (22), 351.0868 (92), 309.0401 (45), 297.0424 (24), 133.0281 (100)	[10, 16]

*Tentatively identified based on the fragmentation pattern in the MS/MS experiments.

was identified as demethoxy CFL-B [10,16].

Compounds **30** and **32** shared a common fragmentation pathway, suggesting that they may be isomers as well: in particular, compound **32** was identified as CFL-A, which was confirmed by the isolated compound, while compound **30** was tentatively identified as cannflavin C (CFL-C) [10,13,16].

Compound **31** ($[M+H]^+ = 407.1854$) had a main product ion at *m/z* 283.0598, having a precursor ion which differs from that of CFL-A for 30 Da. For these reasons, it was identified as demethoxy CFL-A [10,16, 29].

To the best of our knowledge, compounds **27** and **31** were identified for the first time in this work in PEF from hemp inflorescences.

Finally, other phenolic compounds identified in PEF include compound **13** ($[M+H]^+ = 243.1017$, $[M-H]^- = 241.0867$), which was identified as deoxyrhapontigenin (*4′-O*-methylresveratrol), since the loss of the methoxy and of the methyl moieties generated the main product ions at *m/z* 210.0679 and *m/z* 226.0631 in the negative ion mode [30].

Compounds **14** ($[M+H]^+ = 271.0601$, $[M-H]^- = 269.0452$), **15** ($[M+H]^+ = 301.0706$, $[M-H]^- = 299.0557$) and **16** ($[M+H]^+ = 301.0707$, $[M-H]^- = 299.0558$) were identified as apigenin and diosmetin/chrysoeriol isomers, respectively, since their fragmentation patterns are compliant with those described in literature [16,31].

Compound **17** ($[M+H]^+ = 625.2544$) was identified as cannabisin F, that consists in a *N-trans*-feruloyltyramine dimer [32]. Its fragmentation pattern was in accordance with what is already described in the literature, with the main product ion at *m/z* 325.1068 [25].

Finally, compounds **20** ($[M+H]^+ = 285.0757$, $[M-H]^- = 283.0608$) and **22** ($[M+H]^+ = 375.2166$, $[M-H]^- = 373.2015$) were identified as acacetin and hydroxymatairesinol/nortrachelogenin, respectively, given their fragmentation patterns compliant with those already described in the literature [16,33].

3.3. Purification and identification of cannflavins from hemp

The C-6 prenylated flavonoid CFL-A and CFL-B were isolated from hemp decarboxylated plant material (Carmagnola variety), which is a CBD rich chemotype [17]. Cannflavins were identified using ^{1}H NMR data (see Figs. S1-S2 of the Supporting Information), which were compared with the literature [18].

In detail, both CFL-A and CFL-B retain the same flavonoid scaffold visible from the chemical shift of H-3, H-8 and H-2′ respectively at δ_H 6.69, 6.63, 7.60 ppm, and the doublet 7.00–7.02 ppm of H-5′ and 7.68–7.60 ppm of H-6′ as the only C-3′ methoxy at δ_H 4.00 ppm. The most important difference that led to their discrimination is the substituent at C-6 with CFL-B, having a prenyl moiety (δ_H 5.29 ppm as a triplet) instead of the geranyl moiety of CFL-A (δ_H 5.08 and 5.31 ppm both as a triplet).

3.4. Quantitative analysis of PEF using HPLC-UV

After the chemical characterization, HPLC-UV analysis was performed on PEF in order to quantify its main compounds, i.e. CFL-A, CFL-B, their demethoxy derivatives and *N-trans*-feruloyltyramine. Fig. 2 shows a representative HPLC-UV chromatogram of PEF at 342 nm.

PEF was found to be particularly rich in *N-trans*-feruloyltyramine (17.7 ± 2.2 mg/g), CFL-A (10.7 ± 0.8 mg/g) and CFL-B (8.1 ± 0.3 mg/g). These quantitative data, which are the mean of eight analyses, are compliant with what is already described in literature for raw extracts from hemp inflorescences [10,25,34]. The demetoxy derivatives of CFL-A and CFL-B were present in a lower amount in PEF with respect to their parent compounds (2.7 ± 0.3 and 1.3 ± 0.6 mg/g, respectively). To the best of our knowledge, the two demethoxy derivatives were quantified in hemp extracts for the first time in this study.

3.5. In vitro antiproliferative activity of PEF and pure compounds

The antiproliferative activity of PEF was assessed on both Caco-2 and SW480 colon adenocarcinoma cell lines after 24 and 48 h of exposure (Fig. 3). As depicted in Fig. 3A and B, PEF was able to inhibit Caco-2 and SW480 cell proliferation in a concentration dependent manner. The antiproliferative effect after 24 h of incubation was more pronounced against Caco-2 than SW480 cells, with a calculated IC_{50} values of 4.8 ± 0.2 and 5.7 ± 0.2 μg GAE/mL, respectively (Table 2). As shown in Table 2, after 48 h of treatment no significant differences ($P > 0.05$) were found in the antiproliferative activity of PEF against Caco-2 with respect to 24 h, with a calculated IC_{50} value of 4.3 ± 0.4 μg GAE/mL. On the contrary, the antiproliferative activity of PEF against SW480 significantly increased ($P < 0.05$) after 48 h of treatment respect to 24 h with an IC_{50} value of 3.6 ± 0.2 μg GAE/mL.

The most representative phenolic compounds identified and quantified in PEF, including CFL-A, CFL-B and *N-trans*-feruloyltyramine, were tested for their ability to inhibit cell proliferation in both the adenocarcinoma cell lines. As shown in Fig. 4A and B, CFL-A has the highest antiproliferative activity in Caco-2 and SW480 cells among the pure

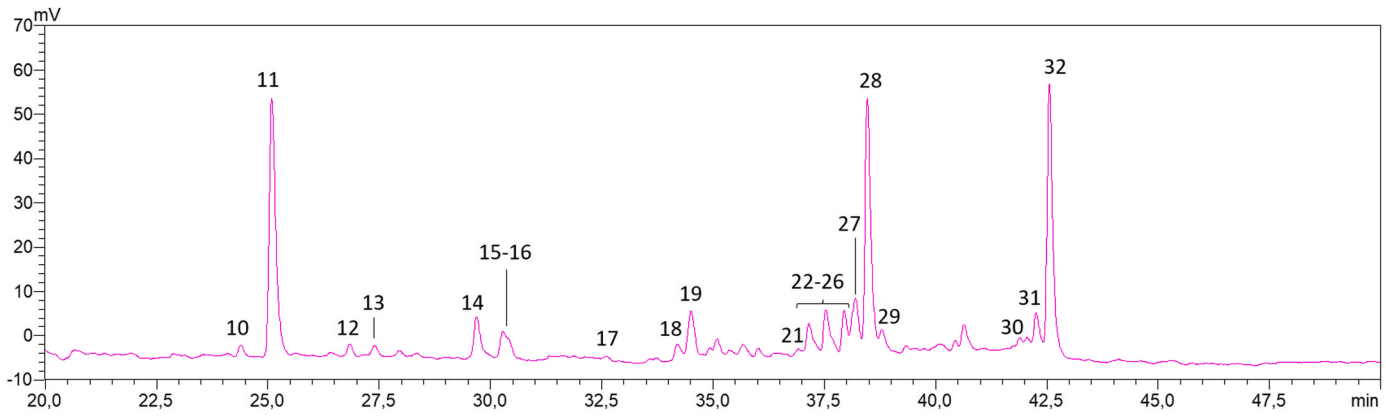

Fig. 2. HPLC-UV chromatogram of PEF at 342 nm. For peak identification, see Table 1.

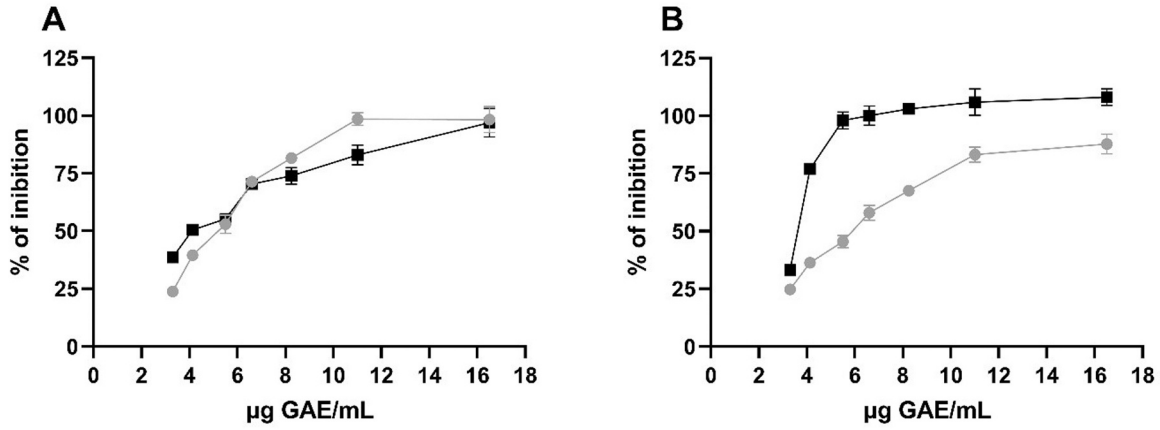

Fig. 3. Antiproliferative activity of PEF against Caco-2 (A) and SW480 (B) colon adenocarcinoma cell lines. Grey lines represent the data collected after 24 h of incubation with PEF at different concentrations, whereas black lines the data collected after 48 h of incubation with PEF at different concentrations. The amount of PEF was expressed as µg GAE/mL.

Table 2
IC_{50} values of PEF and hemp pure compounds after 24 and 48 h of incubation with Caco-2 and SW480 cell lines. Data are expressed as µg GAE/mL for PEF and as µM for pure compounds ± SD.

Compound	Caco-2		SW480	
	24 h	48 h	24 h	48 h
PEF	4.8 ± 0.2^{a}	4.3 ± 0.4^{a}	5.7 ± 0.2^{b}	3.6 ± 0.2^{c}
CFL-A	88.6 ± 4.5^{d}	56.5 ± 2.7^{e}	43.0 ± 2.0 ^{f}	34.7 ± 2.0 ^{g}
CFL-B	n.a.	74.5 ± 3.9 ^{h}	115.7 ± 5.8^{i}	89.3 ± 3.3^{d}
N-trans-feruloyltyramine	n.a.	n.a.	n.a.	n.a.
Cisplatin	78.2 ± 2.0 ^{h}	6.2 ± 0.2^{j}	240.1 ± 9.8^{k}	88.0 ± 5.1^{d}

n.a. means not active compound.
The numbers with the same superscript symbol were not significantly different ($P > 0.05$).

compounds tested. The calculated IC_{50} values (Table 2) were in general lower for SW480 than Caco-2 colon cancer cells, suggesting that this last cell line was less sensitive to this compound. In both these cancer cell lines, the IC_{50} values decreased with the increase of the incubation time. In SW480 cancer cells, CFL-A was more effective than the chemotherapy drug cisplatin, whereas in Caco-2 cells the trend was the opposite (Fig. 4A and B). CFL-B also displayed an antiproliferative activity against SW480, although the effect was lower than CFL-A and, only at 24 h of incubation, higher than cisplatin (Fig. 4A and B). After 48 h of incubation, no significant differences ($P > 0.05$) were found between CFL-B and cisplatin. CFL-B also showed an antiproliferative activity against Caco-2 cells, but only after 48 h of incubation. *N-trans*-feruloyltyramine exhibited no antiproliferative effect on both cancer cell lines after 24 and 48 h of incubation.

Very few studies explored the antiproliferative potential of cannflavins. Tomko et al. (2022) have found that CFL-A was able to inhibit the proliferation of two bladder cancer cell lines with IC_{50} values between 8 and 15 µM after 48 h of incubation [14]. CFL-A exerted its antiproliferative effect by activating apoptosis via caspase 3 cleavage and also displayed synergistic effect with several cannabinoids [14]. An isomer of CFL-B, namely isocannflavin B, has shown an antiproliferative activity on breast and pancreatic cells by promoting cell-cycle arrest and autophagy [13].

From a structural point-of-view the only difference between CFL-A and CFL-B is the length of the group linked to the flavone skeleton. The presence of a geranyl tail in CFL-A, which is longer than the prenyl group of CFL-B, makes this compound more hydrophobic than CFL-B. Recently, González-Sarrías et al. (2022) have demonstrated that hydrophobicity is a crucial parameter in determining the antiproliferative power of stilbenoids in Caco-2 and HT-29 colon cancer cell lines [35]. Therefore, the higher antiproliferative activity observed for CFL-A respect to CFL-B could be due to its higher lipophilic properties. Moreover, the enhanced antiproliferative activity of PEF, with respect to pure cannflavins and cisplatin, may be due to the synergistic effect of its multiple components.

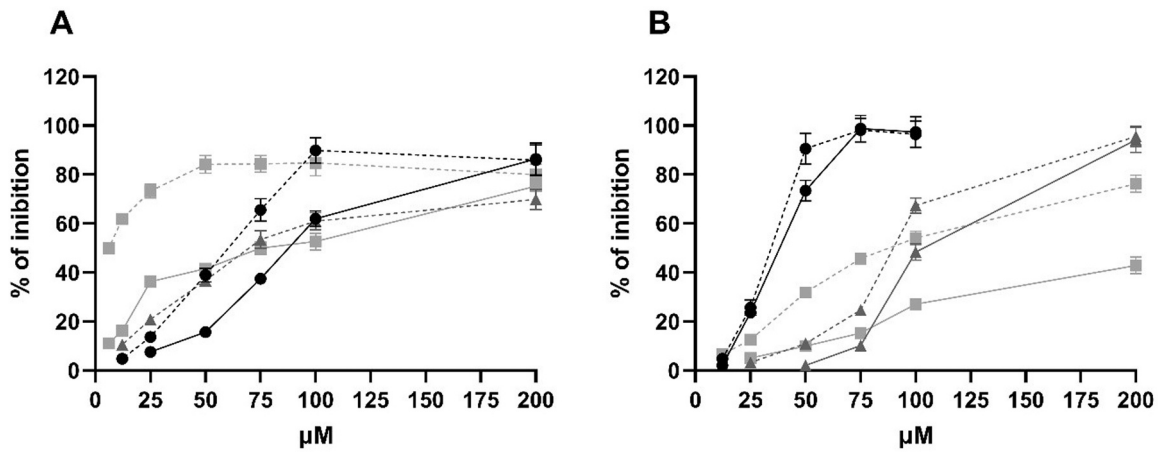

Fig. 4. Antiproliferative activity of pure hemp compounds and cisplatin against Caco-2 (A) and SW480 (B) colon adenocarcinoma cell lines. Tested compounds were CFL-A (black lines with circle), CFL-B (dark grey lines with triangle) and cisplatin (light grey line with square). Solid lines represent the data collected after 24 h of incubation with pure compounds at different concentrations, whereas dotted lines represent the data collected after 48 h of incubation with pure compounds at different concentrations.

4. Conclusions

In this research work, hemp inflorescences were submitted to an extraction procedure, followed by a purification step using preparative flash column chromatography to obtain a fraction highly rich in polyphenols (PEF). To the best of our knowledge, this is the first work with a full chemical characterization of PEF using a new method for untargeted metabolomics based on UHPLC-HRMS, which allowed for the identification of 32 compounds, belonging to different chemical classes of phenolics. The most represented compounds were found to be CFL-A, CFL-B and *N-trans*-feruloyltyramine, as confirmed by HPLC-UV quantitative analysis. Phenolic acids and other phenolic compounds were also detected in PEF.

Based on the results obtained from UHPLC-HRMS and HPLC-UV, the antiproliferative activity of the PEF and pure compounds, isolated from the plant material and identified by ^{1}H NMR, was assessed against Caco-2 and SW480 human colon adenocarcinoma cell lines, after 24 and 48 h of treatment. PEF, in particular, provided interesting IC_{50} values, thanks to the possible synergism of action of its compounds, thus representing a possible new therapeutic product to be further investigate for its bioactivity against CRC.

CRediT authorship contribution statement

Clarissa Caroli: Data curation; Investigation; Methodology; Software; Validation; Visualization; Writing - original draft. **Virginia Brighenti**: Investigation; Methodology; Writing - original draft. **Alice Cattivelli**: Data curation; Investigation; Methodology; Software; Validation; Visualization; Writing - original draft. **Stefano Salamone:** Data curation; Investigation; Methodology; Writing - original draft. **Federica Pollastro**: Data curation; Formal analysis; Funding acquisition; Resources; Supervision; Validation; Visualization; Writing - review & editing. **Davide Tagliazuzzhi**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Resources; Supervision; Validation; Visualization; Writing - review & editing. **Federica Pellati**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Financial support for this work was provided by the Italian Ministry of University and Research (MIUR), which provided the PhD grant of Dr. Clarissa Caroli for the PON research project entitled "*Cannabis sativa* L.: a green and sustainable paradigm of medicinal interest".

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115723.

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