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Multiple pharmacognostic characterization on hemp commercial cultivars: focus on inflorescence water extract activity

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Abstract

One of the most promising economic perspectives of hemp production chain is female inflorescence valorization, despite there being actually no chemical composition or biological data from water fraction. In this context, the focus of this study is the evaluation of protective effects related to hemp water flower extracts from four commercial cultivars (Futura 75, Kc virtus, Carmagnola Cs and Villanova). We evaluated the phytochemical profile through validated spectrophotometric and HPLC methods. Then, we studied the biological activity on C2C12 and HCT116 cell lines, and in an *ex vivo* experimental model of ulcerative colitis, constituted by isolated LPS-stimulated colon. Particularly, we assayed the blunting effects induced by hemp water extract treatment on LPS-induced levels of nitrites, malondialdehyde (MDA), prostaglandin (PG)E_2 and serotonin (5-HT). All tested cultivars displayed similar total phenolic and flavonoid profile. However, Futura 75 water extract displayed a better antioxidant and anti-inflammatory profile. Considering this, Futura 75 extract activity has been subsequently assayed on bacterial and fungal species involved in ulcerative colitis, finding a significant inhibition on *C. albicans* and selected Gram positive and negative bacterial strains.

Concluding, our results support the potential efficacy of hemp inflorecence water extracts in managing the clinical symptoms related to ulcerative colitis.

Keywords: hemp, ulcerative colitis, oxidative stress, inflammation, antimicrobial activity

1. Introduction

Industrial hemp is mostly used for its fruits as a high source of nutrients and fibers from stem, rather than its content in tetrahydrocannabinol (THC).

Traditionally considered a multiuse crop, hemp has been widely cultivated and used throughout history. Actually, economic and pharmaceutical hemp importance is increasing throughout the world, with a global market for low THC valued at $100−2000 millions per year (Montserrat-delaPaz et al., 2014).

Hemp flour, obtained from seeds after oil extraction process, and hemp seed oil are used as ingredients in many certified foods, which have been gaining greater popularity in recent years. Hemp seeds and flour have shown great nutritional value thanks to their content in minerals, vitamins (mostly A, C and E complexes), lipids, proteins and carbohydrates. To this regard, the lipid portion of hemp seeds is very rich (almost 80%) in essential fatty acids used in cell membranes, consisting of a large amount of linoleic (ω-6) and α-linolenic acid (ω-3), often in a 3:1 ratio, ideal for human nutrition and to prevent various pathological conditions, including cancer, cardiovascular, degenerative and inflammatory diseases (Kiralan et al., 2010). These fatty acids are precursors of eicosanoids, deeply involved in homeostatic processes such as inflammation, immunity and vascular tone.

Cannabis sativa THC content typically varies from 3% to 15%, while hemp cultivars are bred to synthesize it only in traces (≤ 0.3% w/w) (De Backer et al., 2012). Despite its versatility, cultivation was prohibited due to the presence of this psychoactive secondary metabolite, found in different parts of the plant, even in the oil. Only varieties of industrial hemp published by EU (Regulation (EC) N° 1251/99 and subsequent amendments) are approved for planting in Europe. These varieties are eligible for cultivation only after the verification of their THC content, which must be less than 0.2% w/w (Regulation EC N°. 1124/2008-12 November 2008) (Da Porto et al., 2014). On the 14th of January 2017, Italian regulation n°172/2017 was published, allowing and regulating hemp production, commerce and therapeutic use in Italy. Different genotypes have been selected and registered through time as well as cultivation methods to avoid the birth of new hybrids.

The fiber isolated from the stalk is used to produce ropes, paper, construction materials, clothing and as a reinforcement in manufacturing composite parts, for example in thermal and acoustic insulation (Vonapartis et al., 2015).

The quantity and quality of hemp fiber production is determined by different factors that should be controlled in order to provide suitable products for a specific use and destination. Harvesting time is one of the most important (Amaducci et al., 2015).

While fiber and seed are hemp main products, there is a growing interest about the valorization of a plethora of hemp secondary metabolites, including terpenes, terpenophenolics, amino acids, fatty acids, sugars, hydrocarbons, flavonoids which could display potential pharmacological effects (Amaducci et al., 2015).

To this regard, hemp essential oil is reported to have an intriguing antimicrobic activity, whereas the whole decocted plant is used against migraine, as a pain reliever and to prevent cognitive decline at very low doses (Nissen et al., 2010).

By contrast, scientific literature lacks on chemical composition or biological activity data from aqueous fraction obtained from industrial hemp female flowers.

Considering that one of the most promising economic perspectives of hemp are female inflorescences, sold dried for technical use, the main focus of the following study is the evaluation of potential protective effects related to aqueous extract from plant female inflorescences, usually considered as a waste material from hemp fiber crops (Bertoli et al., 2010).

In order to investigate and sustain the local market and in view of a more sustainable circular economy, four commercial hemp cultivars, named Futura 75, Carmagnola Cs, Kc Virtus and Villanova have been investigated from a phytochemical, toxicological and pharmacological point of view. Particularly, the water extracts of each cultivar have been assayed for phenolic composition determination and protective effect assessment in a validated *ex vivo* model of ulcerative colitis constituted by isolated rat colon challenged with *E. coli* lipopolysaccharide (LPS) (Menghini et al., 2016; Locatelli et al., 2017a). Finally, multiple herbal preparations, including cannabis extracts, showed potential antibacterial activity on multiple strains, possibly due to the phenolic profile. (Chakraborty et al., 2018; Tănase et al., 2018). In this context, hemp water extracts have been also subjected to a microbiological pilot study to evaluate the putative inhibitory role on specific bacterial strains and fungi involved in ulcerative colitis. The results support the use of hemp flower water extracts as potential source of antioxidants with potential efficacy in managing clinical symptoms related to ulcerative colitis.

2. Materials and Methods

2.1. Pharmacognostic studies

2.1.1. Hemp samples, reagents and standard solutions

Four samples of flowered aerial parts of different *Cannabis sativa* L. cultivars were supplied by Hemp Farm Italia scarl [Tortoreto (TE), IT]. The study was conducted on ‘Futura 75’, ‘Kc virtus’, ‘Villanova’, ‘Carmagnola Cs’ varieties, cultivated under controlled conditions, avoiding chemical

additives and harvested manually. The samples were collected in 2017 and then immediately dried in ventilated oven (40 °C) until constant weight, clumsily chopped and stored in airtight plastic bags, in a darkness and dry place and at room temperature (22-24°C), before performing phytochemical and biological assays.

These products are sold by the company itself as industrial hemp, therefore THC content results < 0.2% w/w, according to the European Regulation EC no. 1124/2008 - 12 November 2008. Every sample has a peculiar content in THC, CBD and CBN, respectively:

- 'Futura 75' - 0.026% w/w, 2.37% w/w, 0.03% w/w;
- 'Kc virtus' - 0.12% w/w, 5.01% w/w, 0.03% w/w;
- 'Villanova' - 0.02% w/w, 3.51% w/w, 0.05% w/w;
- 'Carmagnola Cs' - 0.14% w/w, 4.60% w/w, 0.14% w/w.

Sodium carbonate anhydrous, hydrochloric acid 37%, HPLC-grade n-hexane and ethyl acetate were supplied by Carlo Erba (Milano, IT). Rutin hydrate, gallic acid, aluminum chloride hexahydrate, HPLC-grade methanol and acetonitrile, Folin & Ciocalteau's phenol reagent and acetic acid (glacial) were supplied by Sigma-Aldrich (St. Louis, USA). The ddH_2O for HPLC analysis was obtained using the Millipore Milli-Q Plus water purification system supplied by Millipore Corp. (Bedford, USA), while distilled water was collected long after a chloride treatment has been conducted.

2.1.2. Sample preparation

Each dried cultivar sample was weighed (0.2 g) using a Precisa XT220A supplied by Micro Precision Calibration Inc. (Grass valley, USA) in 50 mL Falcon tubes and then immediately homogenized together with the extraction solvent using a T25 digital Ultra-Turrax device, supplied by IKA (Staufen, GER) for 30 s at 10000 g.

This treatment partially uniformed the grain size in order to improve the yield of the extraction procedure described below.

2.1.3. Ultrasound-assisted extraction (UAE)

UAE of the homogenates was carried out. Distilled water was used as extraction solvent to simulate the possible home-made use (decoction, infusion) of hemp inflorescences.

The sample tubes with the mixture were placed in a Transsonic T460 ultrasonic bath supplied by Elma (Singen, GER) for 10 min. at 60°C and full power (35 kHz).

The solid-liquid ratio and UAE parameters were preliminarily optimized estimating the dry extract yield referred to extraction time and solvent consumption on a single variety of *C. sativa* (Futura 75) and then maintained for the whole study (1:50).

The obtained extracts were immediately centrifuged at 4000g and 4 °C for 5 min using a 5810R centrifuge supplied by Eppendorf (Milano, IT) and the liquid supernatant was collected, adjusted to a final known volume and analyzed.

Three aliquots of each extract were collected and dried to estimate the extraction yield, in terms of dry extract weight.

2.1.4. *Total phenolic and flavonoid content*

Total phenolic and flavonoid contents were also quantified spectrophotometrically and the results were expressed as gallic acid (mg GAE/g extract) and rutin (mg RE/g extract) equivalents. The experimental procedures for all these preliminary assays were comprehensively described in our previous papers (Zengin et al., 2016a; Zengin et al., 2016b).

2.1.5. Polyphenol fingerprint by HPLC-PDA

HPLC-PDA fingerprint of the main phenolics was obtained by means of a validated method using a reversed phase HPLC-PDA in gradient elution mode (Locatelli et al., 2017b; Zengin et al., 2016a).

Analyses were carried out by using a Waters liquid chromatograph equipped with a photodiode array detector, a C18 reversed-phase column (Prodigy ODS (3), 4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA), an on-line degasser (Biotech 4-CH degasi compact, LabService, Anzola Emilia, Italy) and a column oven set at 30 °C (± 1 °C). The gradient elution was achieved by a solution of water–acetonitrile (93:7 ratio, with 3% of acetic acid) as initial conditions. The complete separation was performed in 60 min. The detailed method, alongside with the chromatograms related to analyzed standards and samples, were reported as *Supplementary data*.

2.1.6. *Allelopathy bioassay*

Allelopathy bioassay was conducted in 90 mm diameter Petri dishes, each containing a double layered filter paper disk, previously soaked into the tube containing the plant extract at different concentrations. Distilled water was used as negative control. The pH values of the extracts were measured with the litmus paper, resulting in a value of 6, that is generally the optimal condition for commercial seed growth. This is one of the strongly influencing factors of the process, as well as the liquid volume added to each Petri dish.

The disks were divided into 3 areas, each one added with 10 seeds of the corresponding lettuce variety, previously imbibed into dH_2O for at least 10 min. Seeds were inspected along the sowing process to ensure their uniform size and integrity.

Lettuce is one of the most suitable dicotyledon for this kind of bioassay because of its fast germination rate and high sensitivity. Petri dishes were then sealed with parafilm, to ensure a closed-system model, and incubated in darkness at room temperature.

Bioassay took 3 days and plates were then stored at 4 °C to slow a subsequent growth during the measurement process carried out with a ruler. Seeds were considered germinated only when a root length ≥ 1 mm was observed (Mahmoodzadeh et al., 2015). The number of germinated seeds was noted every day and the length of hypocotyls and roots was taken after the third day of treatment.

2.2.Toxicological, Pharmacological and Microbiological studies

2.2.1. Artemia salina lethality bioassay

Artemia salina cysts were hatched in oxygenated artificial sea water (1g cysts/L). After 24 h, brine shrimp larvae were gently transferred with a pipette in 6 well plate containing 2 mL of hemp extracts at different concentrations (0.1-20 mg/mL) in artificial sea water. Ten larvae *per* well were incubated at 25-28°C for 24h. After 24 h, the number of living napulii were counted under light microscope and compared to control untreated group. Results were expressed as percentage of mortality calculated as: ((T– S)/T)*100. T is the total number of incubated larvae and S is the number of survival napulii. Living nauplii were considered those exhibiting light activating movements during 10 seconds of observation. For each experimental condition two replicates *per* plate were performed and experimental triplicates were performed in separate plates.

2.2.2. *In vitro* studies

Human cardiomyocyte C2C12 and colon cancer-derived HCT116 cell lines were cultured in DMEM (Euroclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1.2% (v/v) penicillin G/streptomycin in 75 cm^2 tissue culture flask (n=5 individual culture flasks for each condition). The cultured cells were maintained in humidified incubator with 5% CO_2 at 37 °C.

For cell differentiation, C2C12 and HCT116 cell suspensions at a density of 1×10^6 cells/mL were treated with various concentrations (10, 50, and 100 ng/mL) of phorbol myristate acetate (PMA, Fluka) for 24 h or 48 h (induction phase). Thereafter, the PMA-treated cells were washed twice with ice-cold pH 7.4 phosphate buffer solution (PBS) to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). Morphology of cells was examined under an inverted phase-contrast microscope.

To assess the basal cytotoxicity of water flower hemp extracts, a viability test was performed on 96 microwell plates, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

Cells were incubated with extracts (ranging in the concentration 10-1000 μg/mL) for 24 h. 10 μL of MTT (5 mg/mL) was added to each well and incubated for 3 h. The formazan dye formed was extracted with dimethyl sulfoxide and absorbance was recorded as previously described (Menghini et al., 2018). Effects on cell viability were evaluated in comparison to untreated control group.

Finally, we tested extracts on HCT116 cell line, in wound healing experimental paradigm. Cell migration was determined using the scratch wound healing assay with slight modification (Ju et al., 2012). HCT116 cells (6×10^3 cells/well) were seeded on 6-well plastic plates. Cells monolayers were preliminarily treated with a proliferation inhibitor mitomycin C (sigma-Aldrich) at the non-toxic concentration of 5 μM, in order to exclude the effect of cell proliferation (Taniguchi et al., 2018). After 2 h on cells in the confluence interval 85-90%, a wound was generated by scratching the cell monolayer using a 0-200 μL pipette tip. Two gentle washes with PBS were performed to remove suspended and damaged cells. Cells were incubated in serum free media supplemented with Hemp extracts at the non-toxic concentration of 100 μg/mL. Cell migration was followed capturing at least 3 microscope images/well at different time points: 0, 24 and 48 h. An inverted light Leika microscope equipped with Nikon 5100 camera was used to capture image at 4x magnification. The quantification of scratch area with no cells was quantified using Image-J software (NIH). Using GraphPad software, mean data at T0, 24 and 48 h were calculated for untreated control and hemp group and expressed as percentage variation with reference to relative 100% at 0 h.

2.2.3. *Ex vivo* studies

Male adult Sprague-Dawley rats (200-250 g) were housed in Plexiglass cages (40 cm × 25 cm × 15 cm), two rats per cage, in climatized colony rooms (22 ± 1 °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00 - 19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g).

Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research.

According to the recognized ethical principles of "Replacement, Refinement and Reduction of Animals in Research", colon specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Local Ethical Committee (University "G. d'Annunzio" of Chieti-Pescara) and Italian Health Ministry (Italian Health Ministry authorization N. 880, delivered on 24th August 2015). Rats were sacrificed by CO_2 inhalation (100% CO_2 at a flow rate of 20% of the chamber volume per min) and colon specimens were immediately collected and maintained in humidified incubator with 5% CO_2 at 37 °C for 4 h, in RPMI buffer with added bacterial LPS (10 µg/mL) (incubation period).

During the incubation period, tissues were treated with scalar sub-toxic concentrations of water hemp extract (100 µg/mL). Tissue supernatants were collected, and the PGE_2 level (ng/mg wet tissue) was measured by radioimmunoassay (RIA), as previously reported (Chiavaroli et al., 2010; Locatelli et al., 2018; Menghini et al., 2016). Briefly, specific anti-PGE_2 was developed in the rabbit; the cross-reactivity against other prostanoids is < 0.3%. 100 µL of prostaglandin standard or sample were incubated overnight at 4 °C with the ^{3}H-prostaglandin (3000 cpm/tube; NEN) and antibody (final dilution: 1:120000), in a volume of 1.5 mL of 0.025 M phosphate buffer. Free and antibody-bound prostaglandins were separated by the addition of 100 µL 5% bovine serum albumin and 100 µL 3% charcoal suspension, followed by centrifuging for 10 min at 4000 x g at 5 °C and decanting off the supernatants into scintillation fluid (Ultima Gold™, Perkin Elmer) for β emission counting. The detection limit of the assay method is 0.6 pg/mL. Additionally, tissue supernatant was assayed for nitrite determination by Griess assay, as previously described (Zengin et al., 2017). Briefly, nitrite production was determined by mixing 50 µL of the assay buffer with 50 µL of Griess reagent (1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v/v). After 10 min incubation at room temperature, the

absorbance at 540 nm was determined and nitrite concentrations were calculated from a sodium nitrite standard curve.

On the other hand, individual colon specimens were dissected and subjected to extractive procedures to evaluate 5-HT and 5HIAA (ng/mg wet tissue) as previously reported (Brunetti et al., 2014; Ferrante et al., 2016). Regarding the 5-HT analysis, tissues were homogenized in ice bath for 2 min with Potter-Elvehjem homogenizer in 1 mL of 0.05 N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite. Thereafter, samples were analyzed by HPLC coupled to electrochemical detection consisting of ESA Coulochem III detector equipped with ESA 5014B analytical cell.

Finally, malondialdehyde (MDA) level was determined through the thiobarbituric acid reactive substances (TBARS) method (Mihara et al., 1980). Briefly, tissue specimens were added with 1% H_3PO_4 and 0.6% thiobarbituric acid, and then incubated at 96 °C for 20 min. Absorbance was recorded at 532 nm, and the MDA level was expressed as g/mL.

2.2.4. Antimicrobial susceptibility testing

In vitro antimicrobial activity of water extracts Futura 75 was assessed against three bacterial strains, namely *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 6538) and two yeasts and filamentous fungi, namely *Candida albicans* (YEPGA 6183) and *C. tropicalis* (YEPGA 6184).

Voucher microbial cultures are maintained in the PeruMycA culture collection of the Department of Chemistry, Biology and Biotechnology (DCBB) (University of Perugia, Italy) and are available upon request.

For Minimum Inhibitory Concentration (MIC) determination, hemp Futura 75 extract ranged from 0.562 to18 mg mL^{-1}. Ciprofloxacin (Sigma Aldrich-Milan, Italy) and fluconazole (Sigma Aldrich-

Milan, Italy) were used as control antimicrobial agents for bacteria and fungi, in the range 0.125-120 µg mL^{-1} (CLSI M100 S21) and 0.063–16 µg mL^{-1} (CLSI 2012; CLSI 2017), respectively.

2.2.5. Antibacterial activity assay

MIC determination was performed according to the broth dilution method M07-A9 drafted by the Clinical and Laboratory Standard Institute (CLSI M07-A9, 2012).

Shortly, working bacterial suspensions (inocula) for MIC determination were prepared as follows: a few colonies from 24 h-old cultures on TSA plates were transferred to Mueller-Hinton broth (MHB) and incubated statically overnight at 37 °C. Cell density of each inoculum was hence adjusted to that of the opacimetric standard Mac Farland 0.5 (1.5×10^8 CFU/ml). 20 µL of bacterial suspensions were used to inoculate 1 mL of MHB medium containing serial dilutions of active plant extracts.

To further assess the viability of bacterial cells at MIC end-points, the tetrazolium salt assay optimized by Sabaeifard and collaborators (2014) was used. Following 20 h incubation for MIC determination, 230 µL of bacterial cultures were collected and transferred to 96-wells plates. Hence, 20 µL of a 2,3,5-triphenyl-tetrazolium chloride (TTC) solution were added to each well in order to reach a final concentration of 0.4%. Controls consisted of MHB-grown bacterial cultures (viability controls) and uninoculated MHB with plant extracts (incubation controls). 96-wells plates were incubated for 6 h at 37 °C prior to measure absorbance at 405 nm in a Tecan Infinite 200 PRO spectrophotometer (Tecan Trading AG, Switzerland).

2.2.6. Antifungal activity assay

Susceptibility testing against yeasts and filamentous fungi was performed according to the CLSI M27-A3 and M38-A2 protocols, respectively (CLSI 2008a,b; CLSI 2012b; CLSI 2017).

RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma) with L-glutamine and without sodium bicarbonate, supplemented with 2% glucose (w⁄v), buffered with 0.165 mol L^{-1} morpholinepropanesulphonic acid (MOPS), pH 7.0, was used throughout the study.

Briefly, the inoculum suspensions were prepared from 7-day-old cultures grown on Sabouraud Dextrose Agar (SDA; Difco) at 25 °C and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (Mac Farland standard). Filamentous fungi and yeasts inoculum suspensions were diluted to a ratio of 1:50 in RPMI 1640 to obtain twice an inoculum size ranging from 0.2 to 0.4 × 10^{4-5} CFU mL^{-1}. This was further confirmed by plating serial dilutions of the inoculum suspensions on SDA.

MIC end-points (µg mL^{-1}) were determined after 24 h (for *C. albicans* and *C. tropicalis*) of incubation in ambient air at 30°C (CLSI 2012, CLSI 2017).

For the plant extracts, the MIC end-points were defined as the lowest concentration that showed total growth inhibition (Pagiotti et al. 2011). The MIC end-points for fluconazole were defined as the lowest concentration that inhibited 50% of the growth when compared with the growth control (CLSI 2012, CLSI 2017).

Geometric means and MIC ranges were determined from the three biological replicates to allow comparisons between the activities of plant extracts.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Gaussian distribution of data was assessed by D'Agostino and Pearson omnibus normality test. Data were means ± SEM and analyzed by Kruskal-Wallis test followed by Mann-Whitney *post-hoc* test. Statistical significance was set at $p < 0.05$. As regards the animals randomized for each experimental group, the number was calculated on the basis of the "Resource Equation" N=(E+T)/T (10≤E≤20) (Charan and Kantharia, 2013), according to the guidelines suggested by the "National Centre for the Replacement, Refinement and Reduction of Animals in Research" (NC3RS) and reported on the following web site: https://www.nc3rs.org.uk/experimental-designstatistics. In particular, N is the number of animals

per treated group. E represents the degrees of freedom of the analysis of variance (ANOVA). T is the number of treatments. Considering that E values should be between 10 and 20, the animal number N for *ex vivo* analysis was chosen in accordance to an E value of 20.

3. Results and discussion

3.1.Phytochemical and phytotoxic profile

Spectrophotometric analyses showed that water flower extracts from Futura 75, Kc Virtus and Villanova cultivars displayed a quite similar total phenol and flavonoid content (Tables 1). On the other hand, in Carmagnola Cs cultivar water extract, the concentration of these metabolites seems to be about half the content of the other cultivars.

The punctual phenol profile assessed by HPLC-PDA analysis showed analogue results. All the cultivar extracts displayed a similar phenolic profile with some exceptions. Rutin content revealed to be higher in Kc Virtus cultivar extract. Additionally, Kc Virtus was the only cultivar showing detectable and measurable coumaric acid level. On the other hand, benzoic acid level was more significant in Futura 75, despite there being a higher total benzoic acid content in Villanova extract (Table 2). In agreement with the spectrophotometric total phenol assessment (Table 1), Carmagnola Cs revealed a poorer panel of phenols, compared to the others.

As an early approach to study their biological activity, hemp extracts have been tested in an allelopathy model. Effects of sowing on substrates imbibed with scalar extract concentrations (1:1; 1:2; 1:5; 1:10, diluted in distilled water) have been evaluated. The assay has been conducted on 3 varieties of commercial lettuce seeds (Trocadero, Iceberg and Lollo bionda). During the experiments we evaluated the effects of the extracts on seeding germination of growth. After challenging the seeds with flower extracts, in all diluted samples we did not observe any significant effect on seed growth and germination (Figures 1-3), thus giving a preliminary index of extract biocompatibility for the following microbiological and pharmacological assessments.

3.2.Toxicological and Pharmacological profile

As a preliminary approach to evaluate potential toxicity, hemp extracts, in the concentration range 0.1-20 mg/mL, were tested on brine shrimp lethality assay. It is a typical and general bioassay that could give information on bioactivity of complex plant extracts evaluated as lethality induced on the brine shrimp, *Artemia salina* Leach. This organism is commonly used to investigate a variety of biological and toxicological activities of plant extracts and is considered, at least partially, predictive of cytotoxicity (Ohikhena et al., 2016). Experimental procedure was conducted following previous published data, with slight modification (Taviano et al., 2013). The hemp extracts did not reveal any toxicity in the concentration range (0.1-20 mg/mL), with LC_{50} values in the range 1.156-2.696 mg/mL (Figure 4)

The resulting LC_{50} value has been indicatory to choose the extract concentration, at least 10 fold lower (100 µg/mL), for the subsequent evaluation of the effects on C2C12 and HCT116 cell line viability (MTT test).

All the tested cultivar extracts (100 µg/mL) confirmed a good biocompatibility, as revealed by the null effect on both cell line viability (Figures 5). Additionally, when we tested the extracts (100 µg/mL) in an experimental model of wound healing, in HCT116 cells, all the extracts resulted ineffective in modifying the spontaneous cell migration up to 48 h following treatment (Figure 6), thus ruling out a possible role of the extracts in modifying migration and invasion capacities of HCT116 human colon cancer cells. Taken together, these preliminary toxicological findings suggested the concentration 100 µg/mL as a good biocompatibility limit for the following pharmacological evaluations.

Particularly, we performed a subsequent panel of experiments on isolated rat colon specimens challenged with LPS. To this regard, estimation of nitrite level is a useful marker of the synthesis of NO and could potentially be used as an indicator of disease activity in chronic inflammatory conditions, including ulcerative colitis (Goggins et al., 2001). NO is a well-known free radical

which can react with a variety of biomolecules in body fluids and tissues. These interactions produce a number of oxidation products including nitrite, nitrate, nitrosyl (NO-heme) species, and S- and N-nitroso products. The level of these NO-related substances, in fluids and tissues, is assumed to reflect the activity of NO-synthases, including the inducible NO synthase (iNOS) which is expressed at high levels during inflammation (Saijo et al., 2010).

We observed that all the extracts were able to reduce LPS-induced nitrite level, in isolated rat colon (Figure 7). Actually, the downregulation of nitrite level induced by the extracts is consistent with their total phenolic and flavonoid content (Raihan et al., 2009). This could explain, albeit partially, the minor efficacy exerted by Carmagnola Cs extract, which displayed a total phenolic and flavonoid content significantly lower than the other extracts.

Consistently with this evidence, we observed that hemp water extracts displayed a significant reduction of LPS-induced increase in tissue MDA (Figure 8), a recognized biomarker of lipid peroxidation (Ferrante et al., 2017; Mancuso et al., 2012). Lipid peroxidation has long been involved in tissue damage related to several chronic disease states, including IBDs (Achitei et al., 2013). Macrophages and neutrophils are the main production sites of ROS/RNS whose effects include neutrophil recruitment at the inflamed epithelial colon tissue. Actually, this effect is consistent with the elevated concentration of phenolics found in the extract (Table 1). Phenolics and particularly flavonoids were found to be effective in reducing the burden of oxidative stress, in the colon of mice intraperitoneally injected with LPS, *via* suppressing phosphorylation in mitogen-activated protein kinases (MAPKs) pathway, which is crucial for macrophage activation and the production of inflammatory mediators (Lin et al., 2010). In agreement with the observed minor antioxidant effect, we found that Carmagnola Cs water extract revealed the less effective in preventing lipid peroxidation in LPS-challenged colon specimens.

Additionally, we evaluated the effects of the extracts on LPS-induced PGE_2 and 5-HT, two key pro-inflammatory mediators in the colon.

Prostaglandin E_2 (PGE_2) is generated by cyclooxygenase 2 (COX-2) conversion of arachidonic acid. Accordingly with the present findings, the reduced levels of PGE_2 could account for the anti-inflammatory effects induced by hemp water extracts (Figure 9). Particularly, Villanova extract revealed the most effective in reducing LPS-induced PGE_2 levels, in rat isolated colon. Actually, this could be partially related to its major content in catechin (Singh and Katiyar, 2011), compared to Carmagnola Cs, Futura 75 and Kc virtus.

On the other hand, Futura 75 and Kc virtus were the most effective in blunting LPS-induced 5-HT steady state levels, in rat colon (Figure 10). Actually, the inhibitory effects exerted by these two cultivars could be related to multiple concomitant mechanisms. On one side rutin, which is more expressed in Kc Virtus extract, resulted able to reduce 5-HT release *in vitro* (Chen et al, 2002). On the other hand, benzoic acid has been reported to stimulate 5-HT turnover (Batshaw et al., 1988). In despite of a more detailed assessment of pharmacological modulation of neurotransmitter release, given by isolated neuronal ending experimental paradigm, several studies confirmed that steady state tissue 5-HT concentration proves to be a valuable index of neurotransmitter activity, including synthesis and release (Bungo et al., 2009; Clark et al., 2006). 5-HT pro-inflammatory role in ulcerative colitis has been previously suggested (Regmi et al., 2014), possibly involving the activation of 5-HT3 receptors (Mousavizadeh et al., 2009). In this context, the reduction of 5-HT and PGE_2 tissue levels further supports the protective role related to hemp water extract treatment.

All the cultivar exerted multiple protective effects in LPS toxicity model. On the other hand, Futura 75 cultivar displayed a good inhibitory effect against all the tested biomarkers, whereas the other cultivars sometimes resulted ineffective.

To this regards, we performed a final set of experiments to evaluate the effects of Futura 75 on Gram positive and negative bacterial strains and fungal species deeply involved in ulcerative colitis (Guo et al., 2015; Iguidbashian et al., 2018; Trojanowska et al., 2010; Wang et al., 2018).

Regarding the bacterial strain used, *Futura 75* water extract was particularly effective against *S. aureus* (3.57 mg mL^{-1}) and to a lesser extent towards *E. coli* and *P. aeruginosa* (7.14 mg mL^{-1}) (Table 3). Whereas Table 4 shows the MIC ranges and geometric means of plant extracts and fluconazole against the fungal species tested. *C. albicans* was the most sensitive fungus to Futura 75 extract and fluconazole. Actually, we hypothesize that the antibacterial and antifungal activity of Futura 75 water extract could be related, albeit partially, to the total phenolics and flavonoids (Bottari et al., 2017; de Camargo et al., 2017).

Concluding, the use of commercial hemp cultivar, particularly Futura 75 water extract, as food supplementation, could provide significant improvements for health, as revealed by the downregulating effects on oxidative stress- and inflammation-related biomarkers, but also against pathogen bacterial and fungal strains, including *S. aureus* and *C. albicans*.

Nevertheless, considering the limits of the *in vitro* and *ex vivo* assessments, further *in vivo* approaches are required in order to confirm both efficacy and biocompatibility of water hemp extracts. Finally, in agreement with the accepted principle of "Circular Economy", our findings further support an intriguing approach to innovatively improve the hemp chain production by considering new health applications for THC-free inflorescences which could be regarded as a strategy to implement the economic efficacy of hemp fiber crop, validating a potential waste as high quality byproducts with promising pharmaceutical applications

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Conflict of interest

Authors declare no financial/commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

References:

Achitei D, Ciobica A, Balan G, Gologan E, Stanciu C, Stefanescu G. Different profile of peripheral antioxidant enzymes and lipid peroxidation in active and non-active inflammatory bowel disease patients. Dig Dis Sci. 2013;58(5):1244-9.

Amaducci S, Scordia D, Liu FH, Zhang Q, Guo H, Testa G, Cosentino SL. (2015). Key cultivation techniques for hemp in Europe and China. Industrial Crops and Products 2015;68:2–16.

Batshaw ML, Hyman SL, Coyle JT, Robinson MB, Qureshi IA, Mellits ED, Quaskey S. Effect of sodium benzoate and sodium phenylacetate on brain serotonin turnover in the ornithine transcarbamylase-deficient sparse-fur mouse. Pediatr Res. 1988;23(4):368-74.

Bertoli A, Tozzi S, Pistelli L, Angelini LG. (2010). Fibre hemp inflorescences: From crop-residues to essential oil production. Industrial Crops and Products 2010;32:329–337 .

Bottari NB, Lopes LQ, Pizzuti K, Filippi Dos Santos Alves C, Corrêa MS, Bolzan LP, Zago A, de Almeida Vaucher R, Boligon AA, Giongo JL, Baldissera MD, Santos RC. Antimicrobial activity and phytochemical characterization of Carya illinoensis. Microb Pathog. 2017;104:190-195.

Brunetti L, Orlando G, Ferrante C, Recinella L, Leone S, Chiavaroli A, Di Nisio C, Shohreh R, Manippa F, Ricciuti A, Vacca M. Peripheral chemerin administration modulates hypothalamic control of feeding. Peptides. 2014;51:115-21.

Bungo T, Shiraishi J, Yanagita K, Ohta Y, Fujita M. Effect of nociceptin/orphanin FQ on feeding behavior and hypothalamic neuropeptide expression in layer-type chicks. Gen Comp Endocrinol. 2009;163(1-2):47-51.

Chakraborty S, Afaq N, Singh N, Majumdar S. Antimicrobial activity of Cannabis sativa, Thuja orientalis and Psidium guajava leaf extracts against methicillin-resistant Staphylococcus aureus. J Integr Med. 2018 Sep;16(5):350-357.

Charan J, Kantharia ND. How to calculate sample size in animal studies? J Pharmacol Pharmacother. 2013;4(4):303-6.

Chen WM, Jin M, Wu W. [Experimental study on inhibitory effect of rutin against platelet activation induced by platelet activating factor in rabbits]. Zhongguo Zhong Xi Yi Jie He Za Zhi. 2002;22(4):283-5.

Chiavaroli A, Brunetti L, Orlando G, Recinella L, Ferrante C, Leone S, Di Michele P, Di Nisio C, Vacca M. Resveratrol inhibits isoprostane production in young and aged rat brain. J Biol Regul Homeost Agents. 2010;24(4):441-6.

Clark KA, MohanKumar SM, Kasturi BS, MohanKumar PS. Effects of central and systemic administration of leptin on neurotransmitter concentrations in specific areas of the hypothalamus. Am J Physiol Regul Integr Comp Physiol. 2006;290(2):R306-12.

CLSI, 2008a. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2008b. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2012. Reference method for broth dilution antifungal susceptibility testing of yeasts; 4th informational supplement. CLSI document M27-S4. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2017. Performance standards for antifungal susceptibility testing of filamentous fungi. 1st. ed. CLSI supplement M61. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA, 2012.

Da Porto C, Decorti D, Natolino A. Separation of aroma compounds from industrial hemp inflorescences (*Cannabis sativa* L.) by supercritical CO_2 extraction and on-line fractionation. Industrial Crops and Products 2014; 58:99–103.

De Backer B, Maebe K, Verstraete AG, Charlier C. Evolution of the content of THC and other major cannabinoids in drug-type cannabis cuttings and seedlings during growth of plants. J Forensic Sci. 2012;57(4):918-22.

de Camargo AC, Regitano-d'Arce MAB, Rasera GB, Canniatti-Brazaca SG, do Prado-Silva L, Alvarenga VO, Sant'Ana AS, Shahidi F. Phenolic acids and flavonoids of peanut by-products: Antioxidant capacity and antimicrobial effects. Food Chem. 2017;237:538-544.

Ferrante C, Orlando G, Recinella L, Leone S, Chiavaroli A, Di Nisio C, Shohreh R, Manippa F, Ricciuti A, Vacca M, Brunetti L. Central inhibitory effects on feeding induced by the adipo-myokine irisin. Eur J Pharmacol. 2016;791:389-394.

Ferrante C, Recinella L, Locatelli M, Guglielmi P, Secci D, Leporini L, Chiavaroli A, Leone S, Martinotti S, Brunetti L, Vacca M, Menghini L, Orlando G. Protective Effects Induced by Microwave-Assisted Aqueous Harpagophytum Extract on Rat Cortex Synaptosomes Challenged with Amyloid β-Peptide. Phytother Res. 2017;31(8):1257-1264.

Goggins MG, Shah SA, Goh J, Cherukuri A, Weir DG, Kelleher D, Mahmud N. Increased urinary nitrite, a marker of nitric oxide, in active inflammatory bowel disease. Mediators of inflammation 2001;10:69-73.

Guo M, Ding S, Zhao C, Gu X, He X, Huang K, Luo Y, Liang Z, Tian H, Xu W. Red Ginseng and Semen Coicis can improve the structure of gut microbiota and relieve the symptoms of ulcerative colitis. J Ethnopharmacol. 2015 Mar 13;162:7-13.

Iguidbashian JP, Parekh JD, Kukrety S, Andukuri VG. Campylobacter jejuni and Pseudomonas coinfection in the setting of ulcerative colitis. BMJ Case Rep. 2018 Jun 6;2018. pii: bcr-2018-224941.

Ju J, Kwak Y, Hao X, Yang CS. Inhibitory effects of calcium against intestinal cancer in human colon cancer cells and Apc(Min/+) mice. Nutr Res Pract. 2012;6(5):396-404.

Kiralan M, Gül V, Metin Kara S. Fatty acid composition of hemp seed oils from different locations in Turkey. Spanish Journal of Agricultural Research 2010;8(2): 385-390.

Lin YC, Huang DY, Chu CL, Lin WW. Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of toll-like receptors-mediated JNK signaling pathway. Mol Immunol. 2010;47(7-8):1569-78.

Locatelli M, Ferrante C, Carradori S, Secci D, Leporini L, Chiavaroli A, Leone S, Recinella L, Orlando G, Martinotti S, Brunetti L, Vacca M, Menghini L. Optimization of Aqueous Extraction and Biological Activity of *Harpagophytum procumbens* Root on Ex Vivo Rat Colon Inflammatory Model. Phytother Res. 2017a;31(6):937-944.

Locatelli M, Macchione N, Ferrante C, Chiavaroli A, Recinella L, Carradori S, Zengin G, Cesa S, Leporini L, Leone S, Brunetti L, Menghini L, Orlando G. Graminex Pollen: Phenolic Pattern, Colorimetric Analysis and Protective Effects in Immortalized Prostate Cells (PC3) and Rat Prostate Challenged with LPS. Molecules. 2018;23(5). pii: E1145.

Locatelli M, Zengin G, Uysal A, Carradori S, De Luca E, Bellagamba G, Aktumsek A, Lazarova I. Multicomponent pattern and biological activities of seven *Asphodeline* taxa: potential sources of natural-functional ingredients for bioactive formulations. J Enzyme Inhib Med Chem. 2017b;32(1):60-67.

Mahmoodzadeh H, Ghasemi M, Zanganeh H. Allelopathic effect of medicinal plant *Cannabis sativa* L. on *Lactuca sativa* L. seed germination. Acta agriculturae Slovenica 2015;105: 233-239.

Mancuso C, Barone E, Guido P, Miceli F, Di Domenico F, Perluigi M, Santangelo R, Preziosi P. Inhibition of lipid peroxidation and protein oxidation by endogenous and exogenous antioxidants in rat brain microsomes in vitro. Neurosci Lett. 2012;518(2):101-5.

Menghini L, Ferrante C, Leporini L, Recinella L, Chiavaroli A, Leone S, Pintore G, Vacca M, Orlando G, Brunetti L. An Hydroalcoholic Chamomile Extract Modulates Inflammatory and Immune Response in HT29 Cells and Isolated Rat Colon. Phytother Res. 2016;30(9):1513-8.

Menghini L, Leporini L, Vecchiotti G, Locatelli M, Carradori S, Ferrante C, Zengin G, Recinella L, Chiavaroli A, Leone S, Brunetti L, Orlando G. Crocus sativus L. stigmas and byproducts: Qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities. Food Res Int. 2018;109:91-98.

Mihara M, Uchiyama M, Fukuzawa K. Thiobarbituric acid value on fresh homogenate of rat as a parameter of lipid peroxidation in aging, CCl4 intoxication, and vitamin E deficiency. Biochem. Med. 1980;23:302–311.

Montserrat-de la Paz S, Marín-Aguilar F, García-Giménez MD, Fernández-Arche MA. Hemp (Cannabis sativa L.) seed oil: analytical and phytochemical characterization of the unsaponifiable fraction. J Agric Food Chem. 2014 Feb 5;62(5):1105-10.

Mousavizadeh K, Rahimian R, Fakhfouri G, Aslani FS, Ghafourifar P. Anti-inflammatory effects of 5-HT receptor antagonist, tropisetron on experimental colitis in rats. Eur J Clin Invest. 2009;39(5):375-83.

Nissen L, Zatta A, Stefanini I, Grandi S, Sgorbati B, Biavati B, Monti A. Characterization and antimicrobial activity of essential oils of industrial hemp varieties (Cannabis sativa L.). Fitoterapia. 2010;81(5):413-9.

Ohikhena FU, Wintola OA, Afolayan AJ. Toxicity Assessment of Different Solvent Extracts of the Medicinal Plant, Phragmanthera capitata (Sprengel) Balle on Brine Shrimp (Artemia salina). Int. J. Pharmacol. 2016;12:701-710.

Pagiotti R, Angelini P, Rubini A, Tirillini B, Granetti B, Venanzoni R. Identification and characterisation of human pathogenic filamentous fungi and susceptibility to Thymus schimperi essential oil. Mycoses 2011; 54:e364-376.

Raihan SZ, Chowdhury AK, Rabbani GH, Marni F, Ali MS, Nahar L, Sarker SD. Effect of aqueous extracts of black and green teas in arsenic-induced toxicity in rabbits. Phytother Res. 2009; 23(11):1603-8.

Regmi SC, Park SY, Ku SK, Kim JA. Serotonin regulates innate immune responses of colon epithelial cells through Nox2-derived reactive oxygen species. Free Radic Biol Med. 2014 Apr;69:377-89.

Sabaeifard P, Abdi-Ali A, Soudi MR, Dinarvand R. Journal of Microbiological Methods 2014; 105: 134–140.

Saijo F, Milsom AB, Bryan NS, Bauer SM, Vowinkel T, Ivanovic M, Andry C, Granger DN, Rodriguez J, Feelisch M. On the dynamics of nitrite, nitrate and other biomarkers of nitric oxide production in inflammatory bowel disease. Nitric Oxide 2010;22:155-167.

Singh T, Katiyar SK. Green tea catechins reduce invasive potential of human melanoma cells by targeting COX-2, PGE2 receptors and epithelial-to-mesenchymal transition. PLoS One. 2011;6(10):e25224.

Tănase C, Coşarcă S, Toma F, Mare A, Man A, Miklos A, Imre S, Boz I. Antibacterial activities of beech bark (fagus sylvatica l.) polyphenolic extract. Environmental Engineering and Management Journal 2018;17(4):877-884

Taniguchi M, Ochiai A, Namae T, Saito K, Kato T, Saitoh E, Tanaka T. The antimicrobial and anti-endotoxic peptide AmyI-1-18 from rice α-amylase and its [N3L] analog promote angiogenesis and cell migration. Peptides. 2018;104:78-84.

Taviano MF, Marino A, Trovato A, Bellinghieri V, Melchini A, Dugo P, Cacciola F, Donato P, Mondello L, Güvenç A. Juniperus oxycedrus L. subsp. oxycedrus and Juniperus oxycedrus L. subsp. macrocarpa (Sibth. & Sm.) Ball."berries" from Turkey: Comparative evaluation of phenolic profile, antioxidant, cytotoxic and antimicrobial activities. Food and chemical toxicology 2013;58: 22-29.

Trojanowska D, Zwolinska-Wcislo M, Tokarczyk M, Kosowski K, Mach T, Budak A. The role of Candida in inflammatory bowel disease. Estimation of transmission of C. albicans fungi in gastrointestinal tract based on genetic affinity between strains. Med Sci Monit. 2010;16(10):CR451-7.

Vonapartis E, Aubin MP, Seguin P, Mustafa AF, Charron JB. (2015). Seed composition of ten industrial hemp cultivars approved for production in Canada. Journal of Food Composition and Analysis 2015;39: 8–12

Wang XM, Tian G, Duan QJ, Wu DQ, Shao J, Wang TM, Wang CZ. [Therapeutic potential of n-butanol extract of Pulsatilla decoction in a murine model of ulcerative colitis induced by DSS

combined with Candida albicans colonization]. Zhongguo Zhong Yao Za Zhi. 2018;43(14):2979-2984.

Zengin G, Locatelli M, Stefanucci A, Macedonio G, Novellino E, Mirzaie S, Dvorácskó S., Carradori, S, Brunetti L, Orlando G, Menghini L, Ferrante C, Recinella L, Chiavaroli A, Leporini L, Mollica A. Chemical characterization, antioxidant properties, anti-inflammatory activity, and enzyme inhibition of *Ipomoea batatas* L. leaf extracts. International Journal of Food Properties 2017; 20: 1907-1919.

Zengin G, Menghini L, Malatesta L, De Luca E, Bellagamba G, Uysal S, Aktumsek A, Locatelli M. Comparative study of biological activities and multicomponent pattern of two wild Turkish species: *Asphodeline anatolica* and *Potentilla speciosa*. J Enzyme Inhib Med Chem. 2016a;31(sup1):203-208.

Zengin G, Nithiyanantham S, Locatelli M, Ceylan R, Uysal S, Aktumsek A, Selvi PK, Maskovic P. Screening of in vitro antioxidant and enzyme inhibitory activities of different extracts from two uninvestigated wild plants: *Centranthus longiflorus* subsp. *longiflorus* and *Cerinthe minor* subsp. *auriculata*. European Journal of Integrative Medicine 2016b; 8(3): 286-292.

Table and Figure captions

Table 1: Total phenols and flavonoids determined in the water flower extracts. Data are expressed means ± SD referred to gallic acid and rutin equivalents, respectively.

Table 2: Phenolic fingerprint of water hemp extracts. Values are expressed in µg/mg dry extract. Data represent the mean ± SD.

Table 3: Minimum inhibitory concentration (MIC) of plant extracts towards selected bacterial strains.

Table 4: Minimum inhibitory concentration (MIC) of plant extracts towards selected yeasts and filamentous fungal strains.

Figure 1 (A-D): Seedlings germination and growth of Iceberg seeds challenged with Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts. Results are expressed as roots and

hypocotyl (seedling) length ± SD at different concentrations and mean of GP after the third day since the sowing. (Extract concentration: 244.65 ± 30.36 mgdw/gdm).

Figure 2 (A-D): Seedlings germination and growth of Trocadero seeds challenged with Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts. Results are expressed as roots and hypocotyl (seedling) length ± SD at different concentrations and mean of GP after the third day since the sowing. (Extract concentration: 244.65 ± 30.36 mgdw/gdm).

Figure 3 (A-D): Seedlings germination and growth of Lollo bionda seeds challenged with Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts. Results are expressed as roots and hypocotyl (seedling) length ± SD at different concentrations and mean of GP after the third day since the sowing. (Extract concentration: 244.65 ± 30.36 mgdw/gdm).

Figure 4 (A-D): Effects of Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts (0.1-20 mg/mL) on *Artemia salina* Leach viability (Brine shrimp lethality test).

Figures 5 (A-B): Effect of Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts (100 µg/mL) on non-tumoral C2C12 and tumoral HCT116 cell line viability (MTT test).

Figure 6 (A-D): Effect of Futura 75 (A), Kc Virtus (B), Carmagnola Cs (C) and Villanova (D) extracts (100 µg/mL) on HCT116 migration (Wound healing test).

Figure 7: Effect of hemp water extracts (100 µg/mL) on LPS-induced nitrite level (mmoL/g wet tissue) in rat colon specimens. Kruskal-Wallis test, $P<0.01$; *post-hoc*, *$P<0.05$ vs. LPS.

Figure 8: Effects of hemp water extract (100 µg/mL) on LPS-induced malondialdehyde (MDA) production in rat colon tissues challenged with LPS. Kruskal-Wallis test, $P<0.05$; *post-hoc*, *$P<0.05$; vs. LPS.

Figure 9: Effect of hemp water extracts (100 µg/mL) on LPS-induced prostaglandin (PG)E2 level (pg/mg wet tissue) in rat colon specimens. Kruskal-Wallis test, $P<0.05$; *post-hoc*, *$P<0.05$; vs. LPS.

Figure 10: Effect of hemp water extracts (100 µg/mL) on serotonin (5-HT) level (ng/mg wet tissue) in rat colon specimens challenged with LPS. Kruskal-Wallis test, $P<0.01$; *post-hoc*, *$P<0.05$vs. LPS.