Nutraceutical potential of hemp (Cannabis sativa L.) seeds and sprouts

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Abstract

In this study the antioxidant effect of *Cannabis sativa* L. seeds and sprouts (3 and 5 days of germination) was evaluated. Total polyphenols, flavonoids and flavonols content, when expressed on dry weight basis, were highest in sprouts; ORAC and DPPH (*in vitro* assays), CAA-RBC (cellular antioxidant activity in red blood cells) and hemolysis test (*ex vivo* assays) evidenced a good antioxidant activity higher in sprouts than in seeds. Untargeted analysis by high resolution mass spectrometry in negative ion mode allowed the identification of main polyphenols (caffeoyltyramine, cannabisin A, B, C) in seeds and of ω-6 (linoleic acid) in sprouts. Antimutagenic effect of seeds and sprouts extracts evidenced a significant decrease of mutagenesis induced by hydrogen peroxide in *Saccharomyces cerevisiae* D7 strain. In conclusion our results show that *C. sativa* seeds and sprouts exert beneficial effects on yeast and human cells and should be further investigated as a potential functional food.

Keywords: *Cannabis sativa* L., seeds, germination, antioxidant capacity, anti-mutagenic activity, metabolomic fingerprint, functional food.

Abbreviations

ORAC: Oxygen Radical Absorbance Capacity

DPPH: 2,2-diphenyl-1 picrylhydrazyl radical scavenger assay

CAA-RBC: Cellular Antioxidant Activity in Red Blood Cells.

Chemical compounds studied in this article:

DPPH, free radical (PubChem CID: 2735032)

DCFH-DA 2',7'-dichlorofluorescein diacetate (PubChem CID: 104913)

Glutamic acid (PubChem CID: 33032)

Caffeoyltiramine (PubChem CID: 9994897)

Cannabisin A (PubChem CID: 15086398)

Cannabisin B (PubChem CID: 101631692)

1. Introduction

The importance of functional foods, nutraceuticals and other natural diet compounds has been related to health promotion and diseases risk reduction. A functional food is defined as "Natural or processed foods that contain known or unknown biologically-active compounds which in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease" (Martirosyan & Singh, 2015).

Seeds (legumes, cereals, oilseeds, etc) play an important role in the human diet, providing macro and micronutrients, dietary fibers, as well as bioactive compounds with health and diseases-prevention benefits. In the last years, an increasing use of non-conventional foods, as chia, quinoa, flax, canola and hemp seeds was observed, due to their nutritional and beneficial properties among people interested in improving and maintaining their health status by changing dietary habits. It has been recently reported that chia (*Salvia hispanica* L.) seeds have a high antioxidant capacity (Salgado-Cruz, Calderón-Domínguez, Chanona-Pérez, Hernández-Álvarez, Díaz-Ramírez, & Farrera-Rebollo, 2014) and quinoa seeds (*Chenopodium quinoa* Willd.) are an excellent example of functional food lowering the risk of various diseases. In particular, the sprouts of quinoa are 'new' vegetables, which can be used in vegetarian nutrition and in common diet too (Vega-Gálvez, Miranda, Vergara, Uribe, Puente, & Martínez, 2010). Moreover, hemp (*Cannabis sativa* L.), flax (*Linum usitatissimum* L.) and canola (*Brassica napus* L.) seed oils are important to human nutrition due to their favorable polyunsaturated fatty acid content, notably α-linolenic acid and linoleic acid (The & Birch, 2014).

C. sativa is an annual herbaceous plant belonging to Cannabinaceae family and originated in Central Asia, known to have played a historically important role in food, fiber and medicine production. For centuries, it has been considered as one of the most important agricultural crops by providing necessities such as cordage, cloth, food, lighting oil and medicines. *C. sativa* subsp. indica, can contain up to 20% of the psychoactive compound D9-tetrahydrocannabinol (THC), while industrial hemp (*C. sativa* subsp. sativa) is characterized by bioactive molecules and by a low content of THC (Andre, Hausman, & Guerriero, 2016). In most European countries, the current upper legal limit for cultivation of hemp for fiber and seeds production is 0.2% THC on dry basis (Russo & Reggiani, 2013).

Currently, hemp seeds are mainly used as feed but their products (oil, meal, flour, protein powder) are achieving a growing popularity in human nutrition (Andre et al., 2016) as excellent source of nutrients, containing all essential amino acids and fatty acids in sufficient amount and ratio to satisfy the dietary human demand (Werz et al., 2014). In particular, they contain 25%-35% of lipids, 20%-25% of proteins, 20%-30% of carbohydrates, 10%-15% of insoluble fibers and a rich array of minerals such as phosphorus, potassium, sodium, magnesium, sulfur, calcium, iron, and zinc (Callaway, 2004). Hemp seeds are rich in protein (25%) and in vitamin E (90 mg/100 gr) (Callaway, 2004; Rodriguez-Leyva & Pierce, 2010).

Hemp seeds contain more than 30% of oil, more than 80% being polyunsaturated fatty acids, particularly linoleic (ω-6) and α-linolenic (ω-3) acids. There are various benefits attributed to ω-3 including anti-cancer, anti-inflammatory and anti-thrombosis properties, stimulation of general metabolism and promotion of fat burning (Russo & Reggiani, 2013). The ω-6/ω-3 ratio in hemp seed oil is about 3:1, considered optimal for human health (Callaway, 2004). Recent studies reported the beneficial effects of these fatty acids and dietary hemp seed itself on platelet aggregation, ischemic heart disease and other aspects

of cardiovascular health (Rodriguez-Leyva & Pierce, 2010). Moreover, hemp seeds and derivates have been identified as a valuable antioxidant food (Chen et al., 2012) for the positive effects of hemp bioactive molecules; further investigations and clinical trials for possible adverse effects of hemp products in the diet have been suggested (Rodriguez-Leyva, McCullough, & Pierce, 2011).

An imbalance between the production of reactive oxygen species (ROS) and the availability of adequate endogenous antioxidants can cause oxidative stress at cellular level with damaging effects to membranes, proteins, enzymes, and DNA, resulting in the progression of chronic diseases, inflammation and carcinogenesis (Girgih, Alashi, He, Malomo, & Aluko, 2014; Wang, Meckling, Marcone, Kakuda, & Tsao 2011). Antioxidants, such as polyphenols from plant extracts, can protect the organism against free radicals attack by reducing or inhibiting cell damages due to the oxidation of lipids or other biomolecules (Chen et al., 2012). The evaluation of polyphenols antioxidant activity is especially important in *in vivo* studies on functional food (Jurčević et al., 2017).

Many works reported the antioxidant and anti-hypertensive properties of hemp seed peptides and protein hydrolysate (The, Bekhit, Carne, & Birch, 2016; Girgih et al., 2014) and of cold pressed hemp oils measured by DPPH and ORAC assay (Yu, Zhou, & Parry, 2005).

While studies regarding the beneficial effects of hemp seeds, oils and meals are numerous, there are few references concerning the biological activities and the potential health benefits of hemp sprouts. Werz et al. (2014) suggested the use of hemp sprouts as a novel anti-inflammatory hemp food product finding that germination and sprouting processes induced the production of anti-inflammatory compounds (prenylflavonoids cannflavins A and B) while cannabinoids were not present at sprout stage. It is known that germination of plant seeds usually causes the *de novo* synthesis of bioactive phytochemicals that protect the plant from various exogenous challenges. Germination-associated changes in the phytochemical profile of seeds are well documented

(Giorgetti et al., 2017) and could promote the commercial development of sprouts enriched in specific phytochemicals, which can be regarded as health-enhancing ingredients.

The aim of this study was to investigate the antioxidant potential of *C. sativa* L. cv. Futura 75 seeds and sprouts, evaluated *in vitro* by polyphenols, flavonoids and flavonols content as well as DPPH and ORAC assays. The antioxidant and anti-hemolytic properties of seeds and sprouts were estimated in an *ex vivo* erythrocytes system by the cellular antioxidant assay (CAA-RBC) and the hemolysis test. Furthermore, the antimutagenic effects were investigated in the yeast *Saccharomyces cerevisiae* D7 strain. Untargeted analysis by mass spectrometry was applied in the present research over hemp seeds and sprouts. In fact, this new approach enables a fingerprint of the material under investigation, giving a characterization in an easy and quick way without complex time-consuming extractions and separations.

2. Materials and Methods

2.1. Chemicals and reagents

All standards and reagents were of analytical grade. Ethanol, sodium carbonate, sodium hydroxide, metaphosphoric acid, Folin-Ciocalteu reagent, gallic acid, quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-dipheny1-1-picrylhydrazyl (DPPH), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), fluorescein sodium salt and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO). Aluminium chloride was purchased from Carlo Erba (Milan, IT); hydrochloric acid was purchased from Merck (Readington, NJ). Bacto peptone, Bacto-agar, Yeast extract were obtained from Oxoid S.p.A (Milan, Italy).

2.2. Plant material, germination and extraction

In this study, *C. sativa* L. cv. Futura 75 (French monoecious) seeds kindly provided by ASSOCANAPA Carmagnola (Torino) were used. This cultivar has been the most cultivated in Italy in the last five years. Twenty five seeds in three replicates were put in 10 cm diameter Petri dishes containing four layers of Whatman filter paper imbibed with 10 ml water at 24°C in the dark. Seeds and sprouts at 3 and 5 days of germination (T3 and T5) were extracted (see below) and processed for polyphenols, flavonoids, flavonols, DPPH, ORAC, mass spectrometry analysis and *ex vivo* assays.

Extraction was performed according to the Pajak method (Pajak, Socha, Gałkowska, Roznowski, & Fortuna, 2014) with some modifications. One g of seeds and one g of sprouts T3 and T5 was suspended in 10 ml of ethanol 80%, homogenized with Ultraturrax (Kinematica Polytron PT MR 2100), shaken for 3 h in the dark at 4°C, and then centrifuged (Jouan CR31 centrifuge, Newport Pagnell, UK) at 4500 xg for 20 min. For antimutagenesis assays, seeds and sprouts extracts were evaporated under vacuum to remove ethanol. The residue was resuspended in DMSO, filtered using sterile VWR filter (0.2µm) and stored at -20°C until use.

2.3. Dry weight determination

One g (fresh weight, FW) of seeds and sprouts T3 and T5 was weighed, dried in an oven at 102°C overnight to constant weight and weighed again to obtain dry weight (DW).

2.4. Phytochemical characterization

Total polyphenols (TPC), total flavonoids and flavonols were measured in seeds and sprouts according to La Marca et al. (2015) and references therein. In particular, for TPC, 100 µl of extract were added to 3 ml of Folin-Ciocalteau (5-fold diluted with distilled water) and after 6 min of incubation, 2 ml of 20% sodium carbonate solution was added drop wise and stirred. After 1 h of incubation at room temperature (RT), absorbance was determined at 760 nm against a blank. TPC

was estimated through the calibration curve of gallic acid and expressed as mg of gallic acid equivalents (GAE/g FW) and mg GAE/g DW of extract.

For flavonoids, 200 µl of extract were mixed with 60 µl of 5% $NaNO_2$ solution, 800 µl of distilled water and incubated at RT for 5 min. Then, 60 µl of $AlCl_3$ 10% in water were added and incubated for 6 min. Next, 400 µl of 1M NaOH and 480 µl of distilled water were added. The mixture was allowed to stand for 5 min at RT before the absorption was measured at 510 nm against a blank. Flavonoids were estimated through the calibration curve of quercetin and expressed as mg quercetin equivalents (QE/g FW) and mg QE/g DW of extract.

For flavonols, 25 µl of extract were added to 225 µl of 10% ethanol, 1 ml of 2% HCl, and 250 µl 01% HCl in 95% ethanol for 30 min at RT and absorbance was determined at 360 nm against a blank. Flavonols were estimated through the calibration curve of quercetin and expressed as mg quercetin equivalents (QE/g FW) and mg QE/g DW of extract.

2.5. In vitro antioxidant activity

2.5.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity was quantified using the oxygen radical absorbance capacity (ORAC) assay with some modifications as described by Gabriele et al. (2015). AAPH was used as peroxyl radical generator and fluorescein as a probe. The fluorescence decay was read at 485 nm excitation and 514 nm emission using a Victor X3 Multilabel Plate Reader (Waltham, MA). Trolox was used as antioxidant standard. Results were expressed as ORAC units (µmol Trolox equivalents/100 g FW or DW).

2.5.2. DPPH radical scavenging activity

The radical scavenging activity was evaluated using the 2,2-dipheny1-1-picrylhydrazyl (DPPH) assay as described by Giorgetti et al. (2017). The absorbance was recorded at 517 nm and the antiradical activity (ARA) was expressed as percentage of DPPH inhibition using the following

equation: ARA = [1-(As/Ac)] x100, where As is the absorbance of the sample and Ac is the absorbance of control.

2.6. Mass spectrometry analysis

Electrospray measurements were carried out on a LCQ-DECA ion trap and in an Orbitrap Q-Exactive instrument (Thermo Finnigan, Bremen, D) for high resolution mass measurements in negative ion mode. Operating conditions for the ESI source were as follows: spray voltage 4.5 kV; capillary temperature 200°C; sheath gas (nitrogen) flow rate, ca. 0.75 L/min.

MS^n product ion experiments were carried out inside the ion trap by isolating the precursor ion and then by applying a supplementary potential for collision induced dissociations; collision gas: He; collision energy: 20-40% arbitrary units. Where specified, MS^2 experiments were carried out in the higher energy collisionally activated dissociation regime (HCD) inside the collision cell of the Q-Exactive instrument. The mass window was 1 or 2 u. Ethanolic extracts of compounds were diluted with methanol to a final concentration of 1×10^{-4} M and introduced via direct infusion in the ESI source at a flow rate of 5 μL/min.

2.7. Ex vivo antioxidant activity

2.7.1. Preparation of erythrocytes

Erythrocytes were collected from six healthy blood donors upon informed consent for the use of residual blood for research purposes, according to the Italian regulations and, in particular, the regulations of "Fondazione G. Monasterio CNR-Regione Toscana". The samples were not pooled. Human blood samples were collected in ethylene diamine tetra acetic acid (EDTA)-treated tubes and centrifuged for 10 min at 2300 x g at 4°C. Plasma and buffy coat were discarded and erythrocytes were washed twice with PBS pH 7.4.

2.7.2. Cellular antioxidant activity in red blood cells (CAA-RBC) assay

The antioxidant activity of hemp extract was evaluated in an *ex vivo* erythrocytes system as described by Frassinetti et al. (2015) using a modified CAA-RBC assay. Red blood cells were diluted with PBS pH 7.4 (1:100) to have a final number of about 10^5 cells/ml, and incubated for 1 h at 37°C with 20 mg/ml of *C. sativa* seeds and sprouts extracts, or with Trolox, used as a reference antioxidant standard, or with PBS for the blank and control sample. In all samples, an oxidant-sensing fluorescent probe, the DCFH-DA, was added to a final concentration of 15 µM. After incubation, erythrocytes were washed twice to remove excess antioxidants and re-suspended in 1 ml cold PBS. Finally, 180 µl of erythrocytes suspension was transferred to a 96-well microplate and 20 µl of AAPH (peroxyl radical generator) were added to a final concentration of 1.2 mM in all wells with the exclusion of blank sample. The fluorescence generated upon oxidation of DCFH-DA was read at 485 nm excitation and 535 nm emission by using a Victor TM X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). Each value was expressed as CAA (cellular antioxidant activity) units, according to Wolfe and Liu (2007) formula: CAA unit = $100-(\int SA / \int CA) \times 100$, where $\int SA$ is the integrated area under the sample curve and $\int CA$ is the integrated area under the control curve (AAPH only-treated cells).

2.7.3. Erythrocytes oxidative hemolysis

Hemolysis of human erythrocytes was generated by thermal decomposition of AAPH in peroxyl radicals as previously described by Frassinetti et al. (2015). Briefly, an erythrocytes suspension at 5% was incubated with PBS (control), or pre-incubated with Trolox (used as antioxidant standard) and the natural extract at 37°C for 1 hour, followed by 4 h incubation with 50 mM AAPH at 37°C. Then, samples were centrifuged 5 min at 1000 x g. The erythrocytes oxidative hemolysis was spectrophotometrically evaluated at 540 nm as released hemoglobin in the supernatant. Each value was expressed as hemolysis percentage with respect to control (AAPH-treated erythrocytes).

2.8. Antimutagenesis assay in yeast cells

The diploid D7 strain of the yeast *S. cerevisiae*, obtained from Zimmermann (Zimmermann, Ker, & Rasenberg, 1975) was used for the evaluation of antimutagenic and protective effects of hemp seeds and sprouts extracts against H_2O_2-induced mutagenesis. Mitotic gene conversion (GC) and point reverse mutation (PM) were measured, at the *trp-5* (tryptophan) and at the *ilv1-92* (isoleucine) loci, respectively. Yeast cells from a maintenance culture were inoculated in 4 ml complete liquid medium (20% glucose, 2% bactopeptone, 1% yeast extract) at concentration of about $2x10^7$ cells/ml. Cultures were supplemented with different concentrations of hemp extracts ranging from 0.10 to 2 mg/ml (data not shown) and incubated under shaking at 30°C for about 6 h to reach the logarithmic growth phase. Aliquots of these cultures were counted and plated, after suitable dilutions, on complete and selective media to ascertain survival, *trp* convertants and *ilv* revertants. Results are expressed as average of three independent experiments.

To measure the susceptibility of *S. cerevisiae* wild-type cells to H_2O_2, cell survival, GC and PM were evaluated in exponential phase cells treated with H_2O_2 concentrations from 1 to 15 mM for 2 h (data not shown) and the concentration of 13 mM was used in antimutagenesis assays. For antimutagenesis evaluation, cells were pre-treated 2 h with non cytotoxic concentration of hemp extracts (1 mg/m1) at 30°C with shaking. Cells were harvested, H_2O_2 was added to a 13 mM final concentration and the mixture was further incubated at 30°C for other 4 h, in order to reach the logarithmic growth phase. After treatment, cells were counted and plated as described above to determine survival, *trp* convertants and *ilv* revertants.

2.9. Statistical analysis

Results of the *in vitro* (ORAC and DPPH) and *ex vivo* (CAA-RBC and antimutagenesis-yeast) assays are the average (± standard deviation) of at least three independent experiments, and each experiment was performed in triplicate for each treatment. Results were statistically

analysed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc- test using Graphpad Prism, version 4.00 for Windows (GraphPad Software). A p value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Dry weight determination

A rapid and progressive water absorption during seeds imbibition and germination of *C. sativa* cv. Futura 75 was observed, so that the ratio DW/FW decreased significantly from 0.95 g DW/g FW in seeds to 0.24 g DW/g FW in T3 and 0.19 g DW/g FW in T5. The determination of water content in plant tissues may allow the expression of molecules on a DW basis, to avoid the misinterpretation of the nutritional significance and health benefit of the material due to the exclusive expression on FW basis (Lim, 2012).

3.2. Phytochemical content

Seeds and sprouts extracts were analyzed by spectrophotometric methods for total polyphenol, flavonoid and flavonols. The values were expressed both as content on FW and on DW basis.

Fig.1A presents the total polyphenol content in analyzed extracts of seeds and sprouts at T3 and T5. The total phenolic content (TPC) of seeds extract was 2.21 ± 0.08 mg GAE/g FW and 2.33 ± 0.07 mg GAE/g DW. The TPC values of sprouts at T3 were 1.21 ± 0.01 mg GAE/g FW and 5.04 ± 0.04 mg GAE/g DW, whereas at T5 were 1.2 ± 0.02 mg GAE/g FW and 6.16 ± 0.057 mg GAE/g DW. When expressed on DW basis, the total phenolic content increased with germination, with highest values at T5.

An increase in phenolic content during germination was reported for several species (Cevallos-Casals, & Cisneros-Zevallos, 2010; Giorgetti et al., 2017) but the majority of studies concerning polyphenol content of *Cannabis* sp. focused on hemp oils or hemp meal and no data are available on hemp sprouts.

The total flavonoids content in hemp seeds and sprouts was reported in Figure 1B. The total flavonoids content of seeds extract was 2.8 ± 0.2 mg QE/g FW and 2.93 ± 0.23 QE/g DW. In sprouts at T3 flavonoids were 1.06 ± 0.2 mg QE/g FW and 4.4 ± 0.9 mg QE/g DW, whereas at T5 were 1.03 ± 0.15 mg QE/g FW and 5.32 ± 0.8 mg QE/g DW. In accordance with total polyphenols content, when expressed on DW basis, also flavonoids increased with germination, with highest values at T5. Our results agreed with those reported by Dueñas, Hernández, Estrella & Fernández (2009) who observed an increase of flavonoids in lupin sprouts during germination and also with those reported by Pajak et al. (2014) who observed that germination increased the flavonoids content in sunflowers, broccoli and radish.

Total flavonols, reported in Fig. 1C, had the same trend during germination as flavonoids with highest values at T5, when expressed on DW basis. More precisely, in seeds extract, total flavonols were 0.8 ± 0.05 mg QE/g FW and 0.85 ± 0.05 QE/g DW. In sprouts at T3 flavonoids were 0.54 ± 0.05 mg QE/g FW and 2.28 ± 0.18 mg QE/g DW, whereas at T5 were 0.48 ± 0.02 mg QE/g FW and 2.45 ± 0.06 mg QE/g DW.

Our results indicated that during germination total polyphenols, flavonoids and flavonols of *C. sativa* significantly increased when expressed on DW basis. The high content of these phytochemical compounds can encourage the consumption of hemp sprouts in the diet.

3.3. In vitro antioxidant activities

3.3.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

In this study, we investigated the in vitro antioxidant capacity and the radical scavenging activity of hemp seeds and sprouts extracts using ORAC assay. The ORAC method uses a biologically relevant free radical source (peroxyl radicals) which is the most prevalent free radical in human biology. The ORAC assay considers both inhibition time and inhibition degree of free radical action caused by antioxidants.

As presented in Fig. 1D, hemp seeds extract showed 127 ± 5 μmol TE/g FW and 134 ± 3 μmol TE/g DW; sprouts extracts exhibited values of 56 ± 0.8 μmol TE/g FW and 239 ± 2 μmol TE/g DW at T3 and 48 ± 1 μmol TE/g FW and 243 ± 3 μmol TE/g DW at T5. A significantly higher antioxidant activity was observed in hemp sprouts compared to seeds, when expressed on DW basis. Recent literature data reported antioxidant activity determined by ORAC assay of hemp protein hydrolysates (The, Bekhit, Carne, & Birch, 2016) giving evidence that the hydrolysis by different protease preparations could produce small peptides that exert antioxidant effects. Same authors previously demonstrated that hemp, flax and canola seeds cakes (by-product of cold pressed oil) possessed high antioxidant capacity, canola having the highest values followed by hemp (The, Bekhit, & Birch, 2014)

The present research on hemp seed and sprouts emphasizes the increase of antioxidant activity with the germination.

3.3.2. DPPH radical scavenging activity

The extracts of *C. sativa* seeds and sprouts were analysed in respect to their antioxidant activity against DPPH radicals. The antioxidant activity of seeds expressed as percentage of DPPH inhibition (ARA %) was 40 ± 2 %, whereas that of sprouts was 52 ± 1 % at T3 and 48 ± 3 % at T5, as shown in Fig. 1D.

The increase in the antioxidant activity during the germination process was in agreement with previous works in which DPPH analysis in seeds and sprouts extracts of radish, broccoli and sunflowers (Pajak et al. 2014) and black kale (Giorgetti et al. 2017) evidenced a general higher antioxidant capacity in young sprouts when compared to the seeds in function of plant species. The enhancement of antioxidant values during germination was supposed to be related to the rise in the content of antioxidant compounds, such as vitamins, polyphenols, flavonols and flavonoids.

The correlation between total polyphenols content and antioxidant activity was evaluated. The analysis evidenced a significant linear correlation between the total polyphenols content and the

antioxidant activity evaluated by ORAC and DPPH (R= 0.935 and 0.88, respectively). A significant linear correlation was also observed between ORAC and DPPH values, with R= 0.98.

Literature data indicate that the antioxidant activity is closely related to polyphenols which have strong antioxidant activities. A significant correlation between phenolic concentration and antioxidant activity evaluated by DPPH method was also reported (Pajak et al., 2014; Chen et al., 2012). Not only the total content but also the type of phenolic compounds (chemical structure, number and position of hydroxyl group, nature of substitutions on the aromatic rings) play a very important role in antioxidant activity (Kolniak-Ostek, 2016).

3.4. Mass spectrometry analysis

The ESI(–) spectra of seeds and sprouts T3 and T5 are reported in Fig. 2. The formation of negative ions of analytes (M) can occur owing to deprotonation ($[M–H]^-$) or by halide addiction, *e.g.* chloride ($[M+Cl]^-$), this latter well evidenced by the isotopic cluster. In the ESI(–) spectra of seeds and sprouts of hemp both these species have been observed.

The ESI(–) spectrum of seeds (Fig. 2A) showed a lot of abundant ions with respect to sprouts at both stages of development (Fig. 2 B,C).The lowest *m/z* ions were attributable to amino acids, such as aspartate, glutamine, glutamic acid and to their derivatives (Table 1). Ions at *m/z* 262.05766 have been assigned to the formula $[C_9H_{12}O_8N]^-$ and attributed to *N,N*-bis(carboxymethyl)glutamic acid. The MS/MS product ion spectrum reported in Fig. 3A was in agreement with the presence of four carboxylic groups as evidenced by consecutive losses of CO_2 and CH_3COOH. Fatty acids, *e.g.* C(18:3) linolenic acid, C(18:2) linoleic acid, C(18:1) oleic acid and C(18:0) stearic acid, were also detected (Table 1). Ions at *m/z* 298.1082 were in good agreement with the formula $[C_{17}H_{16}O_4N]^-$ and assigned to caffeoyltyramine, whose MS/MS product ion spectrum is reported in Fig. 3B.

Organic acids, such as malic acid, and different carbohydrates, *i.e.* di-, tri- and tetrasaccharides, were detected both as $[M-H]^-$ and $[M+Cl]^-$ ions. With the approach followed in this work it was not

possible to assign the stereochemistry and structure to the single species. Their hydrate and phosphate forms were also detected.

Some distinctive compounds already reported in *C. sativa* (Brenneisen, 2007 and references therein; Andre et al., 2016) were found in seeds (Fig. 2A). Ions at *m/z* 357.20743, assigned to the formula $[C_{22}H_{29}O_4]^-$ (error 0.8 ppm) may be attributed to cannabichromenic (CBCA), and/or cannabidiolic (CBDA), and/or cannabicyclolic (CBLA), and/or Δ9-tetrahydrocannabinolic (Δ9-THCA) acids (Table 1).

As all of them are constitutional isomers with the same chemical formula, they could not be separated by this approach. Some of these compounds were already reported in *C. sativa* in function of variety, tissue or of growth stage considered (Andre et al., 2016). In particular, CBDA is the most prevalent phytocannabinoid in the fiber type hemp (Andre et al., 2016). The product of CBDA decarboxylation (CBD), showed many pharmacological properties, such as anti-inflammatory, anti-arthritic, and anti-psychotic effects (Burstein, 2015). It has also been reported that CBD was a potential therapy against neurodegenerative diseases (Andre et al., 2016) and an inhibitor of breast cancer cell migration (Takeda et al., 2012). Similar effects were also reported for CBC (Andre et al., 2016). Another typical *Cannabis* component was found at *m/z* 373.20219 which was assigned to the formula $[C_{22}H_{29}O_5]^-$. On the basis of MS^2 and MS^3 product ion spectra (Figs 3C, D) it could be attributed to cannabielsoic acid, a cannabinoid produced by the photo-oxidation of CBD and CBDA.

Ions at *m/z* 593.19284, 595.20696 and 609.22464 were assigned to the formulae $[C_{34}H_{29}O_8N_2]^-$, $[C_{34}H_{31}O_8N_2]^-$, $[C_{35}H_{33}O_8N_2]^-$, respectively, with errors less than 3 ppm, and attributed to cannabisins A, B and C, respectively (Table 1), a class of phenolic amides and lignanamides previously described in *Cannabis* fruits and roots. Ion intensities decreased in the order cannabisin B, C and A. Recent work suggested that cannabisin B had considerable antiproliferative activity being a promising chemopreventive agent against hepatoblastoma disease (Chen et al., 2013). It has been also described that cannabisin B and caffeoyltyramine isolated from hemp seeds exhibited

significant high DPPH scavenging activity and protective effect against *in vitro* oxidation of human low-density lipoprotein (Chen et al., 2012).

In the high *m/z* range several phospholipids were found that in the seeds are mainly represented by phosphatidylinositols (PIs), a class of membrane lipids reported as key components of membrane trafficking (Thole & Nielsen, 2008) and proposed as molecular sensors and regulators of metabolic flux in plants (Boss & Im, 2012). In particular, ions at *m/z* 831.50366, 833.51983, 835.53140, 857.51854, 859.53528, and 861.54956 were attributed to PI C(34:3) PI C(34:2), PI C(34:1), PI C(36:4), PI C(36:3) and PI C(36:2), respectively (Table 1). As an example, the MS^2 and MS^3 product ion spectra of PI C(34:2) are reported in Fig. 4. In many cases (Murphy, 2015 and references therein) the loss of the *sn-2* free acid and ketene yielded the most abundant ions of this type observed in MS^2 spectra, suggesting that these ions could be indicative of the respective fatty acyl group location on the glycerol backbone. It followed that for the detected PI, there was a C(16:0) chain at *sn-1* and a C(18:2) chain at the *sn-2* position. The characterization of this compound was confirmed by the presence of a quite characteristic ion of PI observed at *m/z* 241 and corresponding to a stable cyclic phosphate anion of inositol (Fig. 4). The formation of this ion was initiated by remote site fragmentation with a hydrogen atom rearrangement from the 2' fatty acyl chain and cleavage of the phosphoester bond with elimination of a molecule of water (Murphy, 2015). A further loss of water forms yielded ions at *m/z* 223 (Fig. 4B). The MS^2 product ion spectrum obtained in the HCD regime inside the collision cell in the Q-Exactive instrument (Table 1) showed a more extensive fragmentation and allowed to detect ions with low *m/z* values, undetectable in the MS^2 spectrum obtained in the ion trap owing to the low-mass-cut off. One of this was the diagnostic ion at *m/z* 153 (Fig. 4C) to which a cyclic phosphate anion structure was assigned (Murphy, 2015).

Other phosphatidylinositol derivatives were due to PI C(34:3), PI C(34:1), PI C(36:4), PI C(36:3) and PIC(36:2) (Table 1).

In the ESI(–) spectra of sprouts, abundant ions were at *m/z* 191.01932 assigned to 2,5-didehydro-gluconic acid and/or 2-keto-gulonic acid, based on the MS^2 product ion spectrum (Fig. 2B, C). The latter is a key intermediate in the production of L-ascorbic acid (vitamin C) (Smirnoff, 1996).

Mass spectra of sprouts were dominated by ions at *m/z* 699.49926 and 723.49511 assigned to the formula $[C_{39}H_{72}O_8P]^-$ and $[C_{41}H_{72}O_8P]^-$, respectively (Fig. 2B, C, Table 1). Based on the molecular formula only, they could be assigned to phosphatidic acids, but a deeper insight of their MS^2 spectra showed that it was not the case. If we consider ions at *m/z* 723 and their MS^2 spectrum, they showed product ions at *m/z* 461, 443 and 279 (Fig. 1S), which could be easily assigned to $[M\text{-}H\text{-}O{=}C{=}CHC_{16}H_{29}]^-$, suggesting the presence of at least one C(18:2) fatty acid residue. Ions at *m/z* 443 were due to a further loss of water, while ions at *m/z* 279 were the fatty acid C(18:2) anions. As no any other fragment ion could be assigned to the loss of another fatty acid chain, it followed that two C(18:2) fatty acid residues were bound to the *sn-1* and *sn-2* positions of glycerol, so the total number of carbon atoms was (18×2)+3=39, although they should have been 41. Therefore an ethyl group should have been located on the phosphate group indicating that these derivatives were not phosphatidic acids but indeed they were phosphatidylethanols (PEths). PEths are a group of aberrant phospholipids formed in cell membranes in the presence of ethanol by the catalytic action of the enzyme phospholipase D on phosphatidylcholine (Nalesso et al., 2011). So it is reasonable that the use of ethanol in the extraction procedure caused the formation of PEths from phosphatidylcholines present in hemp. Other phospholipids found in sprouts are phosphatidylglycerols (PG) PG (34:3) and PG (34:2) at *m/z* 743.48629 and 745.50312, respectively (Table 1).

3.5. Biological effects of hemp seeds and sprouts on human erythrocytes

The antioxidant action in living organisms is not limited to scavenging free radicals but includes up regulation of antioxidant and detoxifying enzymes, modulation of redox cell signalling and gene expression. CAA assay is an important tool for screening antioxidant activity

of natural products, to evaluate their potential to exert an antioxidant response at the cellular level, not just their capacity as a reducing agent (Wolfe & Liu, 2007).

In the present research, the antioxidant properties of hemp seeds and sprouts were evaluated on an *ex vivo* model of human erythrocytes under an oxidative insult in terms of cellular antioxidant activity and oxidative hemolysis inhibition. Erythrocytes play a critical role in antioxidant and anti-inflammatory protection and represent the best cell model for antioxidant screening since they have neither nucleus nor mitochondria; thus, mitochondrial ROS production cannot influence this assay (Honzel, Carter, Redman, Schauss, Endres, & Jensen, 2008). Human erythrocytes were pre-treated for one hour with 20 mg/ml *C. sativa* seeds and sprouts extracts and then exposed to a peroxyl radical generator, the AAPH, to induce a slight oxidative insult. As showed in Fig. 5A, *C. sativa* seeds and sprouts pre-treated erythrocytes exhibited a significantly higher cellular antioxidant activity compared to control, consistent in erythrocytes exposed to AAPH alone (CAA = 0; $p \leq 0.001$), but lower than the Trolox, used as a reference standard (CAA = 94 ± 2). In particular seed extracts pre-treated erythrocytes exhibited the highest cellular antioxidant activity (CAA unit = 82 ± 4) compared to T3 and T5 sprouts with CAA unit values of 34 ± 2 and 42 ± 3.5, respectively. These results suggest a halved scavenging activity of sprouts with respect to seeds and no differences between T3 and T5 sprouts, showing superimposable CAA values independently from days of germination.

Besides, hemp seeds and sprouts extracts were also tested on human erythrocytes exposed to high concentration of peroxyl radical to evaluate the capability to counteract the AAPH-induced oxidative hemolysis. The anti-hemolytic activity of all hemp extracts was compared with that from erythrocytes exposed to AAPH alone (control) responsible for total hemolysis. As shown in Fig. 5B *C. sativa* seeds and sprouts pre-treatment exerted a significant erythrocyte hemolysis inhibition compared to control ($p \leq 0.001$), but lower than Trolox (about 69% hemolysis inhibition), used as a reference standard. Among tested samples, *C.*

sativa seeds exhibited the highest anti-hemolytic effect (about 52% hemolysis inhibition) compared to T3 and T5 sprouts showing about 30% and 39% of hemolysis inhibition, respectively. Our results also demonstrated comparable biological activities between *C. sativa* seeds and Trolox; besides, no differences in anti-hemolytic activity were found between T3 and T5 sprouts.

3.6. Antimutagenesis assay in yeast cells

The eukaryotic yeast *S. cerevisiae* has been used as *ex vivo* cell-based model for medical and medicinal research (Mager & Winderickx, 2005) also useful to study the antimutagenic effects of food derivatives and food extracts. Toxicity of *C. sativa* seeds and sprouts extracts was preliminary tested on *S. cerevisiae* D7. No significant cytotoxic or mutagenic effects were detected at concentrations ranging from 0.1 to 2 mg/ml (data not shown). The concentration of 13 mM H_2O_2 decreased the cell survival and induced significant mutagenic effect.

The data obtained on antimutagenic effect of *C. sativa* seeds extracts on yeast cells, measured as GC and PM, are reported in Figs 5C, D, respectively. As shown in Fig. 5C, the treatment with H_2O_2 induced GC (about 5-fold with respect to the control); in cell cultures pre-treated with hemp seeds and sprouts extracts, the GC values were significantly decreased in respect to H_2O_2 treatment. Similarly, PM values were significantly higher than control (about 2-fold). In cells exposed to H_2O_2, *C. sativa* extracts were able to reduce significantly the mutagenic effect caused by H_2O_2, as shown in Fig. 5D. No statistically significant differences were observed for GC and PM between *C. sativa* seeds and T3 and T5 sprouts.

It is well known that anti-mutagens play an important role in preventing the cell damage that can be induced by oxidative agents and provide a means of slowing the cellular progression towards cancer (Wang, et al., 2011). The unicellular yeast *S. cerevisiae* is a proven model eukaryote for molecular and cellular biology studies (Mager & Winderickx, 2005). The results of our study indicated that seeds, T3 and T5 sprouts extracts of *C. sativa* were able to protect against H_2O_2 induced

cytotoxicity and DNA mutation in *S. cerevisiae*. Since H_2O_2 produces hydroxyl radicals, the most reactive and toxic reactive oxygen species the antimutagenic activity of *C. sativa* could be due to its capability to reduce the intracellular ROS, confirming the obtained results on human erythrocytes. Concerning the biological effects of other plant species, evaluated by these ex vivo assays, a strong antioxidant activity in human erythrocytes and an antimutagenic effect in *S. cerevisiae* was reported on selected lectin free and phaseolamin-enriched variety of common bean (*Phaseolus vulgaris* L.) (Frassinetti et al., 2015).

Conclusions

Seeds and sprouts of *C. sativa* are rich in phytochemical compounds, particularly polyphenols (caffeoyltyramine, cannabisin A, B, C) and also aminoacids and saccharides. In seeds, the pattern of ESI(-) was dominated by the presence of some distinctive compounds modified during germination and no more detectable in sprouts. In particular, main peaks observed in seeds corresponded to some aminoacids with low MW and glutamic acid, gluconic acid, polyphenols caffeoyltyramine, cannabisin A, B, C and several saccharides (disaccharides, trisaccharide and tetrasaccharide).

No differences were observed as main compounds detected in sprouts samples investigated at both times. Among the main compounds detected in sprouts it can be underlined the presence of ω-6 (linoleic acid), of phospholipids and the intermediates in the production of vitamin C (2,5-didehydro-gluconic acid and/or 2-keto-gulonic acid).

In conclusion, the results obtained in this study indicate that seeds and sprouts of *C. sativa* are rich in beneficial bioactive compounds, possess *in vitro* and *ex vivo* antioxidant activity, and also antimutagenic activity on yeast *S. cerevisiae* and they can be used for food purposes.

Declaration of Interest Statement

The authors declare that they have no conflicts of interest.

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Figure captions

Fig. 1. Phytochemical content of *Cannabis sativa* L. seeds and sprouts (T3 and T5) expressed as Fresh/Dry (FW/DW) weight and antioxidant activity. A) total polyphenols (mg GAE/g FW-DW); B) total flavonoids (mg QE/g FW-DW); C) total flavonols (mg GAE/g FW-DW); D) antioxidant activity expressed as µmol TE/g DW (ORAC assay); E) antioxidant activity expressed as ARA% (DPPH assay). Assays were carried out in triplicate and the results were expressed as mean values ± sd (error bars). One-way ANOVA with Bonferroni's multiple comparison *post- hoc* test was performed ($p< 0.05$). Different letters correspond to statistically different values, according to One-way ANOVA.

Fig. 2. Electrospray, ESI(–) mass spectra of ethanol extracts: A) seeds, B) sprouts T3, C) sprouts T5.

Fig 3. ESI(–) product ion mass spectrum of: A) ions at *m/z* 262 with the proposed structure of *N,N*-bis(carboxymethyl)glutamic acid; B) ions at *m/z* 298 with the proposed structure of caffeoyltiramine: C) MS^2 of ions at *m/z* 373 and D) MS^3 of ions $[M\text{-}H\text{-}CO_2]^-$ at *m/z* 329 with the proposed structure of cannabielsoic acid.

Fig. 4. ESI(–) product ion mass spectra of: A) PI (16:0/18:2) $MS^2$and MS^3 of ions at *m/z*833; B) MS^3 of ions $[M\text{-}H\text{-}C_{17}H_{31}COOH]^-$ at *m/z*553, both obtained in the low collision energy regime inside an ion trap; C) MS^2 of ions at *m/z*833obtained in the higher energy collisionally activated dissociation regime (E=25 arbitrary units) inside the collision cell in the Q-Exactive instrument.

Fig. 5. Effects of *C. sativa* L. seeds and sprouts (T3 and T5) extracts on: A) cellular antioxidant activity (CAA) in human erythrocytes; B) AAPH-induced oxidative hemolysis in human erythrocytes. Trolox (500 µM) was used as a reference standard; C) mitotic Gene Conversion; D) Point Reverse Mutation in D7 strain of *Saccharomyces cerevisiae*.

All Assays were carried out at least in triplicate and the results were expressed as mean values ± sd (error bars). One-way ANOVA with Bonferroni's multiple comparison *post- hoc* test was performed. Different letters correspond to statistically different values, according to One-way ANOVA.

References

Andre, C.M., Hausman, J.F., & Guerriero, G. (2016). C*annabis sativa*: The Plant of the Thousand and One Molecules. *Frontiers in Plant Science 7*, 1-17.

Boss, W.F., & Im, Y.J. (2012). Phosphoinositide signaling. *Annual Review of Plant Biology* 63, 409-429.

Brenneisen, R. (2007). Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. In: ElSohly M.A. (Eds) *Marijuana and the Cannabinoids*. Forensic Science and Medicine. Humana Press.

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorganic & Medicinal Chemistry, 23*, 1377-1385.

Callaway, J.C. (2004). Hemp seed as a nutritional resource: An overview. *Euphytica*, *140*, 65-72.

Cevallos-Casals, B.A., & Cisneros-Zevallos, L.C. (2010). Impact of germination on phenolic content and antioxidant activity of 13 edible seed species. *Food Chemistry, 119*, 1485-1490.

Chen, T., He, J., Zhang, J., Li, X., Zhang, H., Hao, J., & Li, L. (2012). The isolation and identification of two compounds with predominant radical scavenging activity in hemp seed (seed of *Cannabis sativa* L.). *Food Chemistry*, *134*, 1030-1037.

Chen, T., Hao, J., He, J., Zhang, J., Li, Y., Liu R., & Li, L. (2013). Cannabisin B induces autophagic cell death by inhibiting the AKT/mTOR pathway and S phase cell cycle arrest in HepG2 cells. *Food Chemistry, 138*, 1034-1041.

Dueñas, M., Hernández, T., Estrella, I., & Fernández, D. (2009). Germination as a process to increase the polyphenol content and antioxidant activity of lupin seeds (*Lupinus angustifolius* L.). *Food Chemistry*, *117*, 599-607.

Frassinetti, S., Gabriele, M., Caltavuturo, L., Longo, V., & Pucci, L. (2015). Antimutagenic and antioxidant activity of a selected lectin-free common bean (*Phaseolus vulgaris* L.) in two cell-based models. *Plant Foods for Human Nutrition, 70*, 35-41.

Gabriele, M., Parri, E., Felicioli, A., Sagona, S., Pozzo, L., Biondi, C., Domenici, V., & Pucci, L. (2015). Phytochemical composition and antioxidant activity of Tuscan bee pollen of different botanic origins. *Italian Journal of Food Science*, *27*, 248-259.

Giorgetti, L., Giorgi, G., Cherubini,E., Gervasi, P.G., Della Croce, C.M., Longo, V., & Bellani, L. (2017). Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of Tuscan black kale *Brassica oleracea* (L.) ssp acephala (DC) var. sabellica L. *Natural Product Research* , DOI:10.1080/14786419.2017.1392953

Girgih, A.T., Alashi, A., He, R., Malomo, S., & Aluko, R.E. (2014). Preventive and treatment effects of a hemp seed (*Cannabis sativa* L.) meal protein hydrolylsate against high blood pressure in spontaneously hypertensive rats. *European Journal of Nutrition*, *53*, 1237-1246.

Honzel, D., Carter, S.G., Redman, K.A., Schauss, A.G., Endres, J.R., & Jensen, G.S. (2008). Comparison of chemical and cell-based antioxidant methods for evaluation of foods and natural products: generating multifaceted data by parallel testing using erythrocytes and polymorphonuclear cells. *Journal of Agricultural and Food Chemistry, 56*, 8319-8325.

Jurcevic, I.L., Dora, M., Guberovic, I., Petras, M., Brncic, S.R., Dikic, D.(2017). Polyphenols from wine lees as a novel functional bioactive compound in the protection against oxidative stress and hyperlipidaemia. *Food Technology and Biotechnology Volume 55, Issue 1,* 109-116.

Kolniak-Ostek, J. (2016). Content of bioactive compounds and antioxidant capacity in skin tissues of pear. *Journal of Functional Foods,* 23, 40–51.

La Marca, M., Pucci, L., Bollini, R., Russo, R., Sparvoli, F., Gabriele, M., & Longo, V. (2015). Antioxidant effect of a fermented powder of Lady Joy bean in primary rat hepatocytes. *Cellular and Molecular Biology Letters*, *20*, 102-116.

Lim, T.H. (2012). Edible Medicinal and Non-Medicinal Plants: Vol. 2, Fruits. eBook ISBN 978-94-007-1764-0.

Mager, W.H., & Winderickx, J. (2005). Yeast as a model for medical and medicinal research. *Trends in Pharmacological Sciences*, *26*, 265–27.

Martirosyan, D.M., & Singh, J. (2015). A new definition of functional food by FFC: what makes a new definition unique? *Functional Foods in Health and Disease*, *5*, 209-223.

Murphy, R.C. (2015). Tandem Mass Spectrometry of Lipids. Molecular Analysis of Complex Lipids, RSC.

Nalesso, A., Viel, G., Cecchetto, G., Mioni, D., Pessa, G., Favretto, D., & Ferrara, S.D. (2011). Quantitative profiling of phosphatidylethanol molecular species in human blood by liquid chromatography high resolution mass spectrometry. *Journal of Chromatography, 1218*, 8423–8431.

Pajak, P., Socha, R., Gałkowska, D., Roznowski, J., & Fortuna, T. (2014). Phenolic profile and antioxidant activity in selected seeds and sprouts. *Food Chemistry*, *143*, 300–306.

Rodriguez-Leyva, D., & Pierce, G.N. (2010). The cardiac and haemostatic effects of dietary hempseed. *Nutrition & Metabolism*, *7*, 32-40.

Rodriguez-Leyva, D., McCullough, R.S, & Pierce, G.N. (2011). Medicinal use of hempseeds (*Cannabis sativa* L.): effects on platelet aggregation. In *Nuts & Seeds in health and disease prevention*. Chapter 74 (pp 637-646), Elsevier Inc.

Russo R., & Reggiani R. (2013). Variability in Antinutritional compounds in Hempseed meal of Italian and French varieties. *Plant*, *1*, 25-29.

Salgado-Cruz, Ma. de la Paz., Calderón-Domínguez, G., Chanona-Pérez, J.J., Hernández-Álvarez, A.J., Díaz-Ramírez, M., & Farrera-Rebollo, R.R. (2014). Chia Seeds: A New Nutraceutical Option. In: Mora-Escobedo, Berrios, Gutierrez-Lopez (Eds). *Seeds as Functional Foods and Nutraceuticals: New Frontiers in Food Science* (pp. 173-190), New York, Nova Science Publisher, Inc.

Smirnoff, N. (1996). The function and metabolism of ascorbic acid in plants. *Annals of Botany, 78,* 661-669.

Takeda, S., Okajima, S., Miyoshi, H., Yoshida, K., Okamoto, Y., Okada, T., Amamoto, T., Watanabe, K., Omiecinski, C.J., & Aramaki, H. (2012). Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration. *Toxicology letters, 214,* 314-319.

The, S.S., & Birch, J. (2014). Effect of ultrasonic treatment on the polyphenol content and antioxidant capacity of extract from defatted hemp, flax and canola seed cakes. *Ultrasonics Sonochemistry*, *21*, 346–353.

The, S.S., Bekhit, A.E-D, & Birch, J. (2014). Antioxidative polyphenols from defatted oilseeds cakes: effect of solvents. *Antioxidants*, 3, 67-80.

The, S.S., Bekhit, A. E-D.Carne, A., & Birch, J. (2016). Antioxidant and ACE-inhibitory activities of hemp (*Cannabis sativa* L.) protein hydrolysates produced by the proteases AFP, HT, Pro-G, actinidin and zingibain. *Food Chemistry*, *203*, 199–206.

Thole, J.M., & Nielsen, E.,(2008) Phosphoinositides in plants: novel functions in membrane trafficking. *Current Opinion Plant Biology*, 11, 620-31.

Vega-Gálvez, A., Miranda, M., Vergara, J., Uribe, E., Puente, L., & Martínez, E.A. (2010). Nutrition facts and functional potential of quinoa (*Chenopodium quinoa* Willd.), an ancient Andean grain: a review. *Journal of the Science of Food and Agriculture, 15,* 2541-2547.

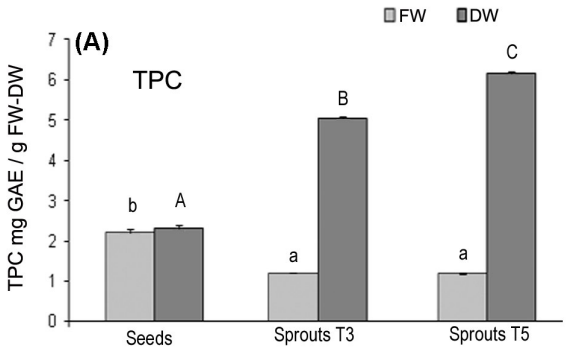
Wang, S., Meckling, K.A., Marcone, M.F., Kakuda, Y., & Tsao, R. (2011). Can phytochemical antioxidant rich foods act anti-cancer agents? *Food Research International*, *44*, 2545-2554.

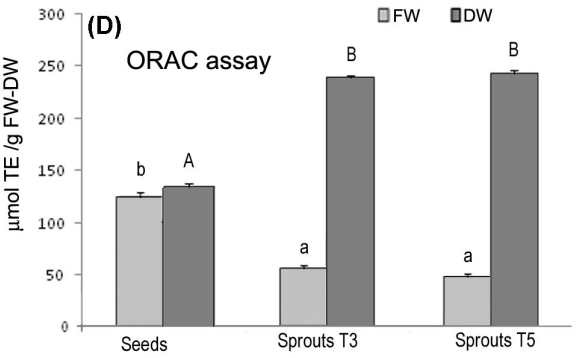
Werz, O., Seerges, J., Shaible, A.M., Weinigel, C., Barz, D., Koeberle, A., Allegrone, G., Pollastro, F., Zampieri, L., Grassi, G., & Appendino, G. (2014). Cannaflavins from hemp sprouts, a novel cannabinoid-free hemp food product, target microsomal prostaglandin E_2 synthase-1 and 5-lypoxygenase. *Pharma Nutrition*, *2*, 53-60.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry, 55*, 8896-8907.

Yu, L.L., Zhou, K.K., & Parry, J. (2005). Antioxidant properties of cold-pressed black caraway, carrot, cranberry, and hemp seed oils. *Food Chemistry*, *91*, 723-729.

Zimmerman, F.K., Ker, N.R., & Rasenberg, C. (1975). A yeast strain for simultaneous detection of induced mitotic crossing-over, mitotic gene conversion and reverse mutation. *Mutation Research*, *28*, 381-388.


(A)
TPC
TPC mg GAE / g FW-DW
FW
DW
b
A
a
B
a
C
Seeds
Sprouts T3
Sprouts T5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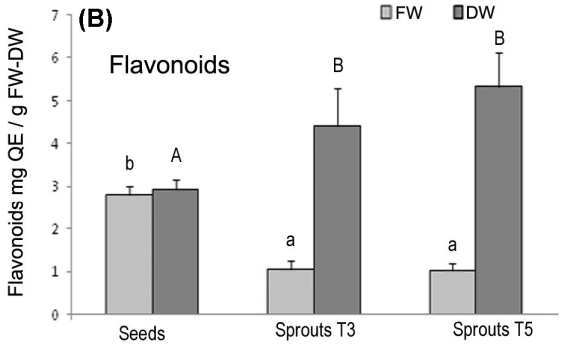
Seeds
Sprouts T3
Sprouts T5


(B)
Flavonoids
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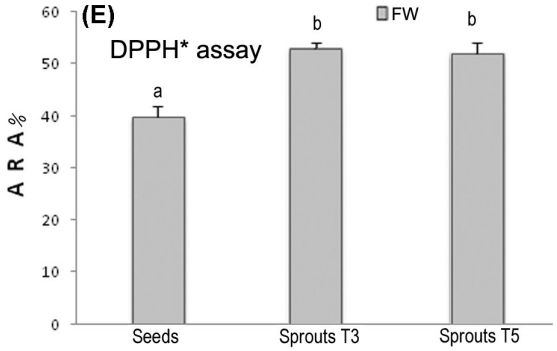
Seeds
Sprouts T3
Sprouts T5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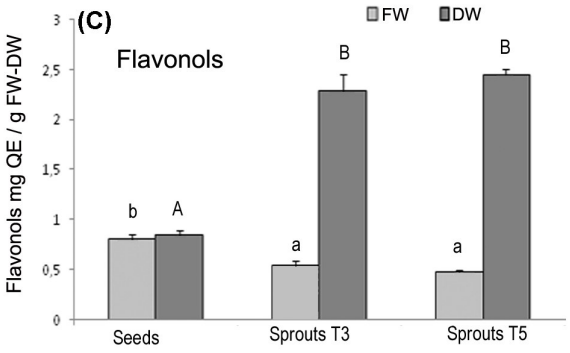
(C)
Flavonols
Flavonols mg QE / g FW-DW
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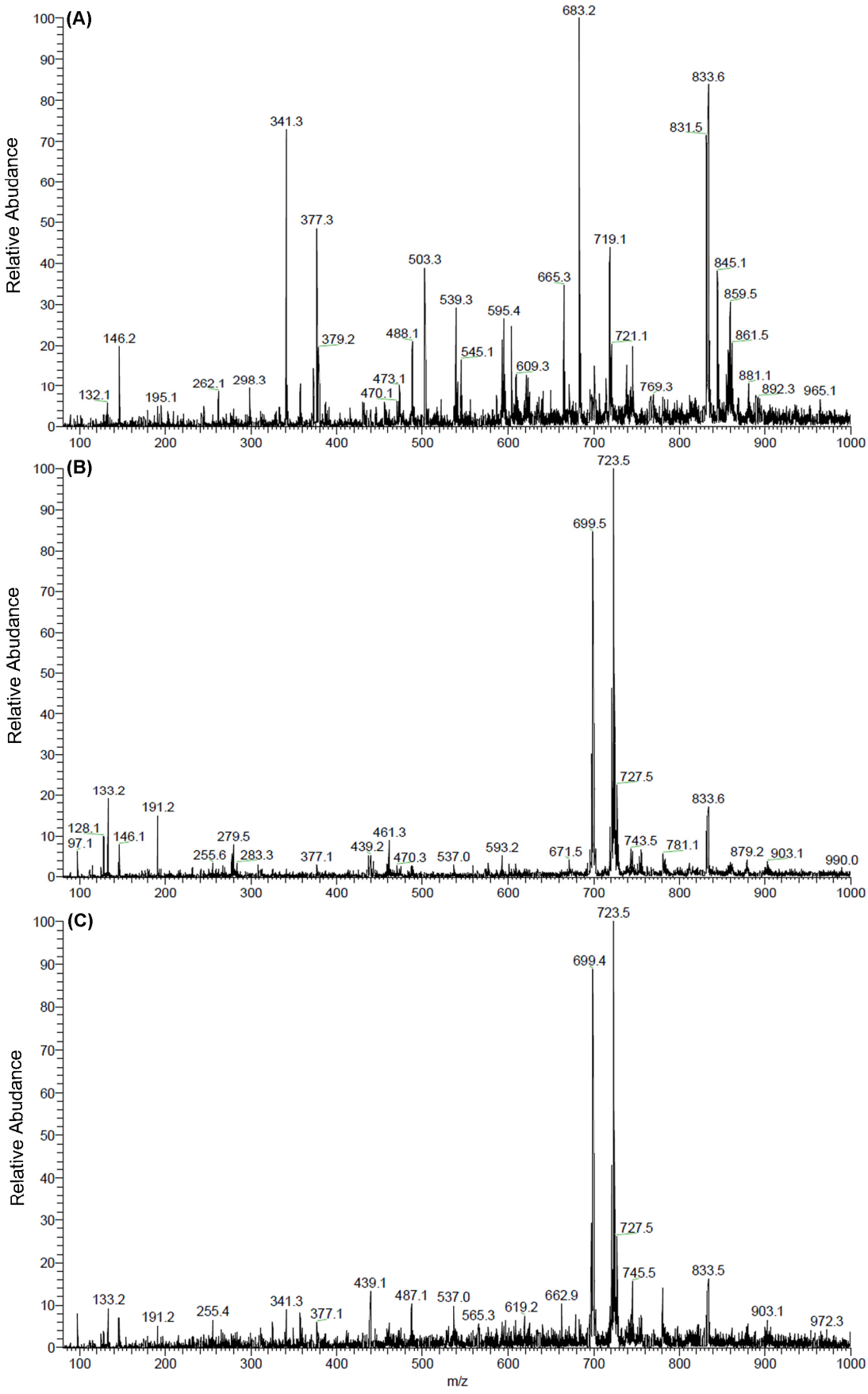
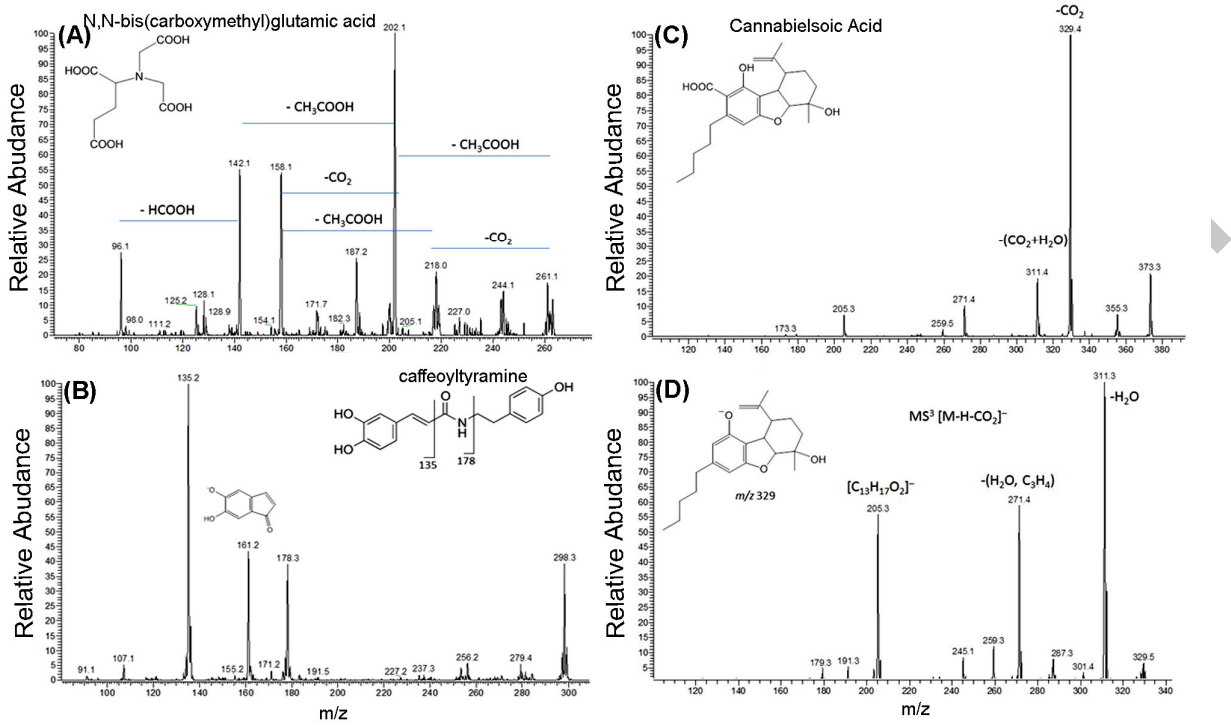
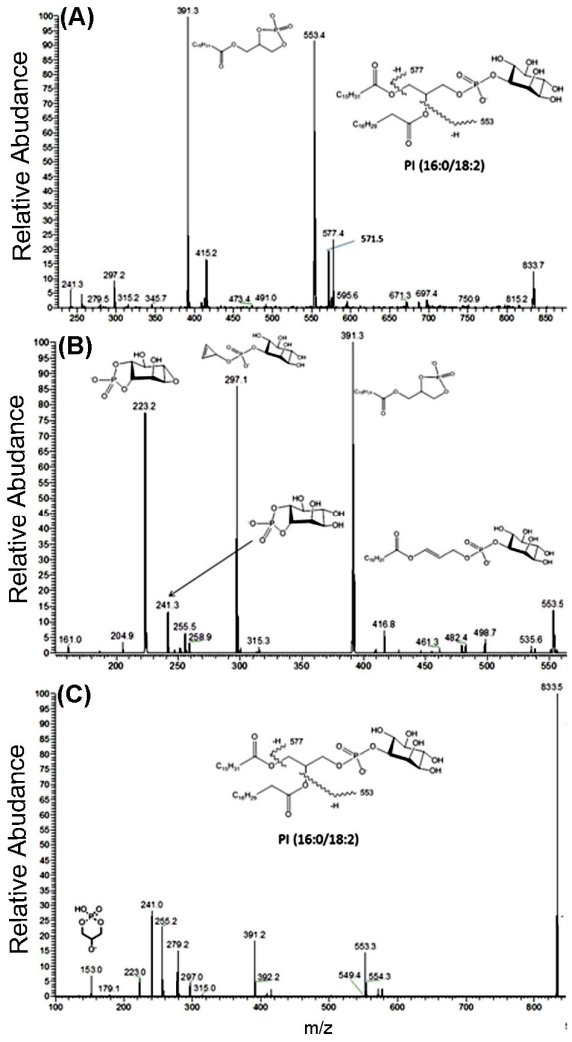
Seeds
Sprouts T3
Sprouts T5







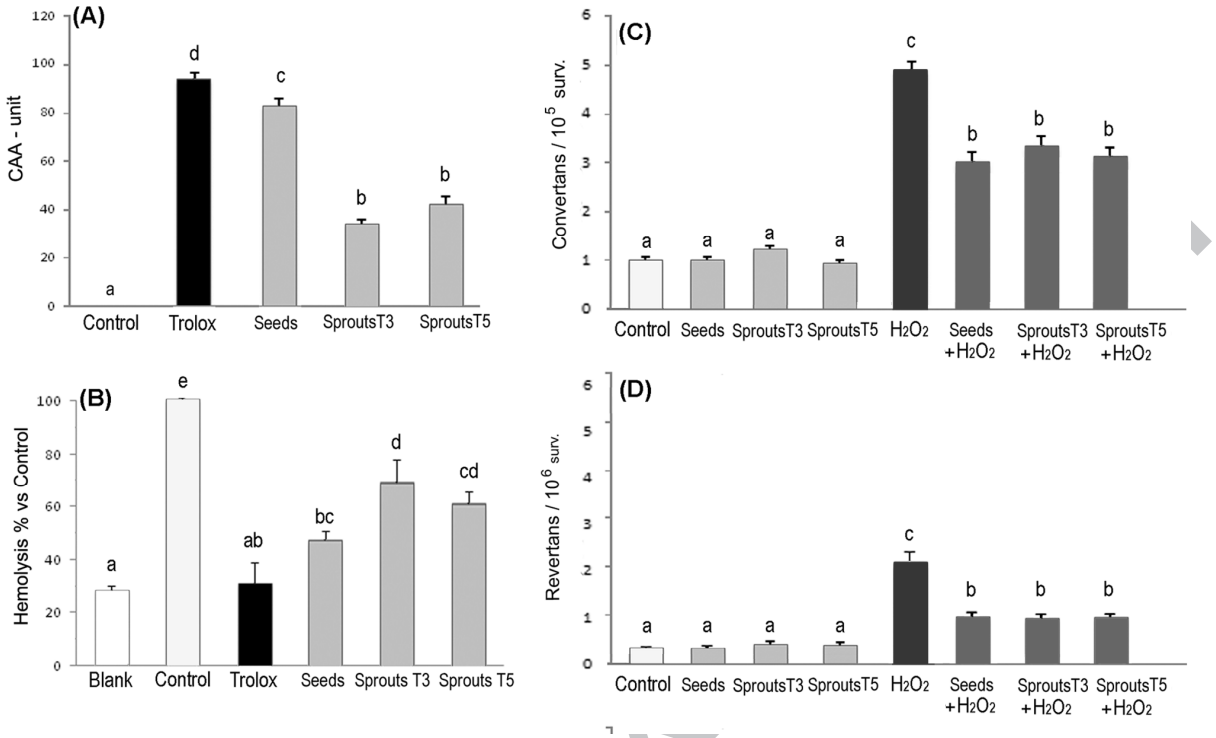


Table 1. Accurate mass, assigned formula, ring-double bond equivalent (RDB), error (ppm), MS^n product ions[a] and attribution of ions found in seed and sprouts of hemp.

Accurate mass (*m/z*)	Formula	RDB	Error (ppm)	MS^n(*m/z*)	Attribution
132.02996	$C_4H_6NO_4$	2.5	-2.052		Aspartate
133.01394	$C_5H_5O_5$	2.5	-2.305	MS^2: 133, 132, 115, 89, 77, 73, 71	Malic acid
145.06145	$C_5H_9O_3N_2$	2.5	-2.830		Glutamine
146.04570	$C_5H_8O_4N$	2.5	-1.240		Glutamic acid
191.01932	$C_6H_7O_7$	3.5	-2.124	MS^2: 191, 129, 111	2,5-didehydro-D-gluconic acid and/or diketo-gulonic acid
262.05766	$C_9H_{12}O_8N$	4.5	3.131	MS^2: 244, 218, 202, 187, 158, 142, 128, 125,96	*N,N*-Bis(carboxymethyl)glutamic acid
277.21738	$C_{18}H_{29}O_2$	4.5	0.276		Fatty acid C18:3 (*e.g.* Linolenic acid)
279.23305	$C_{18}H_{31}O_2$	3.5	0.345		Fatty acid C18:2 (*e.g.* Linoleic acid)
281.24871	$C_{18}H_{33}O_2$	2.5	0.378		Fatty acid C18:1 (*e.g.* Oleic acid)
283.26431	$C_{18}H_{35}O_2$	1.5	0.199		Fatty acid C18:0 (*e.g.* Stearic acid)
298.10823	$C_{17}H_{16}O_4N$	10.5	-0.843	MS^2: 178, 161, 135, 107	Caffeoyltyramine
341.10885	$C_{12}H_{21}O_{11}$	2.5	-0.248		Disaccharide
357.20743	$C_{22}H_{29}O_4$	8.5	0.832	MS^2: 339, 313, 289, 179; MS^3*m/z* 339: 324,295,271, 239, 227	Different Cannabis components: CBCA, CBDA, CBLA, Δ9 THCA

373.20219	$C_{22}H_{29}O_5$	8.5	0.382	MS^2: 329, 311, 271, 205; MS^3*m/z* 329: 287, 271, 259, 245, 205, 191	Cannabielsoic Acid (A and/or B)
377.08541	$C_{12}H_{22}O_{11}Cl$	1.5	-0.537		$[Disaccharide+Cl]^-$
439.08465	$C_{12}H_{24}O_{15}P$	1.5	-2.687	MS^2: 259, 97	Disaccharide hydrate phosphate
503.16187	$C_{18}H_{31}O_{16}$	3.5	0.223		Trisaccharide
539.13847	$C_{18}H_{32}O_{16}Cl$	2.5	0.064		$[Trisaccharide+Cl]^-$
593.19284	$C_{34}H_{29}O_8N_2$	21.5	-0.167		Cannabisin A
595.20696	$C_{34}H_{31}O_8N_2$	20.5	-2.737		Cannabisin B
609.22464	$C_{35}H_{33}O_8N_2$	20.5	0.658		CannabisinC
665.21533	$C_{24}H_{41}O_{21}$	4.5	1.126		Tetrasaccharide
683.22637	$C_{24}H_{43}O_{22}$	3.5	1.792		Tetrasaccharide hydrate
699.49926	$C_{39}H_{72}O_8P$	4.5	3.190	MS^2: 461, 437, 279, 255	PEth(16:0/18:2) (see text)
719.20439	$C_{24}H_{44}O_{22}Cl$	2.5	3.568		$[Tetrasaccharidehydrate+Cl]^-$
723.49511	$C_{41}H_{72}O_8P$	6.5	-2.652	MS^2: 461, 443, 279	PEth(18:2/18:2) (see text)
743.48629	$C_{40}H_{72}O_{10}P$	5.5	-0.764		PG (34:3)
745.50312	$C_{40}H_{74}O_{10}P$	4.5	0.821		PG (34:2)
831.50366	$C_{43}H_{76}O_{13}P$	6.5	0.912		PI (34:3)
833.51983	$C_{43}H_{78}O_{13}P$	5.5	1.533	MS^2: 577, 571, 553, 415, 391, 297, 241;	PI (16:0/18:2)

				MS^3 *m/z* 553: 391, 297, 241, 223 HCD MS^2: 553, 391, 297, 279, 255, 241, 223, 153	
835.53140	$C_{43}H_{80}O_{13}P$	4.5	-3.354		PI (34:1)
857.51854	$C_{45}H_{78}O_{13}P$	7.5	-0.014		PI (36:4)
859.53528	$C_{45}H_{80}O_{13}P$	6.5	1.254		PI (36:3)
861.54956	$C_{45}H_{82}O_{13}P$	5.5	-0.339		PI (36:2)

[a] If not specified, MS^n obtained inside an ion trap.

Abbreviations. CBCA: cannabichromenic acid, CBDA: cannabidiolic acid, CBLA: cannabicyclolic acid, Δ9-THCA: Δ9-tetrahydrocannabinolic acid, PEth: phosphatidylethanol, PG: phosphatidylglycerols, PI: phosphatidylinositol.

Highlights

- *Cannabis sativa* L. seeds and sprouts showed a high *in vitro* and *ex vivo* antioxidant activity.
- *Cannabis sativa* L. seeds and sprouts exerted antimutagenic activity in yeast *Saccharomyces cerevisiae*.
- Phytochemical compounds were characterized and identified via high resolution mass spectrometry in negative ion mode.
- Seeds germination increased the total content of bioactive compounds.
- Hemp seeds and sprouts may represent a potential functional food.