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Research and development of cultivation methods of *Cannabis sativa L.* to maximize the yield of non-THC bioactive compounds of nutraceutical, cosmeceutical and pharmaceutical interest

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Premise

The present Ph.D. thesis has been jointly granted by Regione Marche, the University of Urbino "Carlo Bo" and Opera Investimenti Srl company as part of the Eureka POR Marche FSE 2014/2020 action.

The project pertains to the wide area of sustainable health promotion and improvement of life quality through the development of bioactive botanicals functional foods and nutritional supplements.

The goal of Opera Investimenti Srl – the private partner - was to promote a research program aimed at exploiting legal *Cannabis sativa* cultivation through innovative, highly reproducible and industrially scalable methods, such as aeroponics, to optimize the yield of biologically attractive components in plant roots – usually considered as a waste by-product - and increase the CBD/ Δ^9-THC ratio in the aerial parts.

The three years experimental research can be ideally divided in two sections:

1. Comparison of the growth rate of legal *Cannabis sativa* L. plants cultivated in soil or under aeroponics; isolation of “soil cultivated” and “aeroponics” roots and comparative quali-quantitative characterization of the main bioactive components; effect of elicitation on the yield of bioactives.

2. Evaluation of the biological - namely antioxidant and anti-inflammatory - activity of the ethanolic extract of aeroponics *C. sativa* L. roots and of its major bioactive components.

Abstract

C. sativa has been used for a long time to obtain food, fiber and as a medicinal and psychoactive plant. Today the nutraceutical potential of *C. sativa* is being increasingly reappraised; however, *C. sativa* roots remain poorly studied, despite citations in the scientific literature.

To facilitate roots harvesting and processing, aeroponic (AP) and aeroponic elicited cultures (AEP), have been set up and compared to soil-cultivated plant (SP): interestingly a considerable overgrowth of the plants - particularly of roots - and a significant increase (up to 20-fold in the case of β-sitosterol) in the total content of the above roots' bioactive molecules have been observed in AP and AEP.

Aeroponics, an easy, standardized, free-of-contaminant cultivation technique, allows an ease harvesting/processing of roots along with a greater production of their secondary bioactive metabolites which could be utilized in the formulation of health promoting and health care products**.**

We identified and quantified the presence of valuable bioactives in the extracts of *C. sativa* aeroponic roots (APEX), namely β-sitosterol (ST), friedelin (FR), epi-friedelanol (EFR), stigmasterol and campesterol.

The biological activity of APEX and its major components (ST, FR and, EFR) has been evaluated focusing on the antioxidant and anti-inflammatory properties.

The antioxidant activity has been determined in either acellular (DPPH and Fe^{2+} chelation effect) or cellular settings (cyto- and geno-protection in H_2O_2-treated cells). As to the anti-inflammatory activity, the mRNA inflammatory markers expression and IL-6 levels in LPS-stimulated U937 cells have been studied.

The APEX antioxidant and anti-inflammatory abilities have been further investigated from the functional point of view through the wound healing assay.

Here we report that APEX and its main components showed significant anti-inflammatory and antioxidant activities, which render attractive their

exploitation as natural antioxidant and anti-inflammatory agents, also by virtue of the technical and economic advantages of aeroponic cultivation.

Keywords: *Cannabis sativa* L.; Aeroponics; Roots; β-Sitosterol; Epi-friedelanol; Friedelin; Antioxidant; Anti-inflammatory; Wound healing.

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Introduction

Modern nutrition increasingly involves the consumption of functional foods and nutraceuticals that are useful for health maintenance and for the prevention and treatment of some diseases [1].

Functional foods are solid and/or liquid foods, processed or not, which in addition to nourishing contain also biologically active compounds associated with health benefits. Nutraceuticals - a technical evolution of functional foods - are preparations such as tablets, syrups, powder, etc. that contain active ingredients mostly extracted from plant foods (botanicals); nutraceuticals must provide clinically proven health benefits as well as for the prevention and management and treatment of some chronic diseases [2].

In parallel, the use of health promoting products containing the above bioactive for non-nutritional purposes (skin care, inhalers for respiratory tract, rhinological and otological formulations, eyedrops) is on the rise [3].

C. sativa L. (Linneaus, 1753) is a genus of angiosperms belonging to the Cannabeceae family. According to the prevailing guidelines Hemp includes a single species, called *C. sativa*, which is the most historically widespread plant in the West [4].

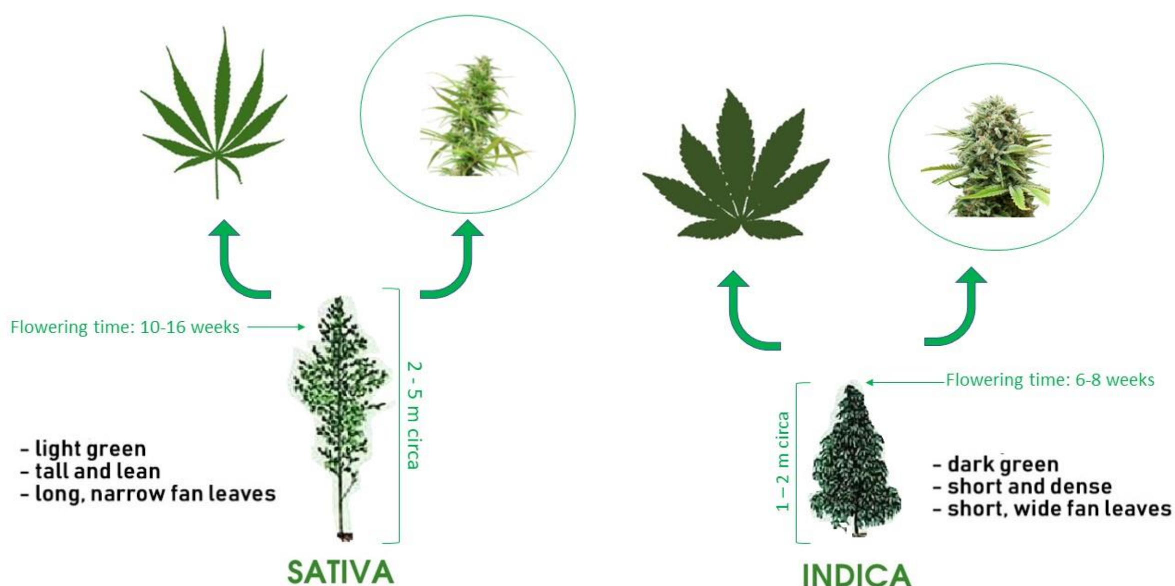


Figure I.1. Subspecies of *Cannabis Sativa* L.

However, the hemp can vary widely in function by pedological factor and assume very different characteristics between them and fit into a separate classification, as in the case of *Cannabis indica.* The variability of secondary metabolites in the plant, determine the taxonomy in two subgroups or chemotypes at depending on the enzyme involved in cannabinoid biosynthesis. Chemotype CBD (cannabidiol) is characterized by the prevalence of the enzyme CBDA-sintetase to which belongs *C. sativa*, for agro-industrial and therapeutic uses, while chemotype Δ^9-THC (Δ^9 -tetrahydrocannabinol), characterized by the enzyme Δ^9-THC A-synthetase present in the varieties of *C. sativa*, used to produce medicines, but is also used for recreational purposes because of the powerful and well-known psychotropic activity [5].

The biological and pharmacological properties of *C. sativa*, have been known since immemorial time to popular medicine, in particular the psychoactive properties that are entirely attributable to the Δ^9-THC content.

In fact, scientific research has confirmed that Δ^9-THC and its analogues to produce the psychotropic effects sought by those who abuse it, also clarifying the

mechanisms of action and interactions with endogenous receptors and specific neuronal pathways [6]. It is obvious that the pharmacological exploitation of *C. sativa*, has been, and still is, severely limited due to high amount of Δ^9-THC content lead in to its potential abuse and drug addiction.

However, recently, the detection of the pharmacological properties of the psychotropic-free metabolite, CBD, and other compounds such as flavonoids, terpenoids and triterpenoids (i.e. FR), and, last but not least, ω3 lipids, has rekindled the attention of the scientific community towards those *C. sativa*, varieties with high CBD content and low Δ^9-THC level.

In addition, some of these substances are present only in the root of the plant, where Δ^9-THC was never detected.

These compounds possess anti-inflammatory, pain relief, antioxidants and regenerative properties [7,8] these activities (or functions) seem more pronounced when the substances are administered together and in the original proportions of the plant through a phenomenon known in pharmacology as "entourage effect".

Consequently, also the agricultural-industrial sector has resumed to invest in the cultivation and exploitation for pharmaceutical, nutraceutical and cosmeceutical purposes of the so-called " *Cannabis* Light ", namely, varieties of *C. sativa* with low Δ^9-THC content (not exceed 0.2%), and therefore for this reason, can be cultivated, processed and marketed.

Furthermore, this *C. sativa* subsp. *sativa* contains numerous bioactive molecules in all parts of the plant [9] and the agricultural industry is interested in developing cultivation methods that also increase the yield of secondary metabolites present in plant parts. For centuries, it has been essential for humans to obtain food, fiber and medicinal compounds. Today the nutraceutical potential of *C. sativa* is being increasingly reappraised [10].

The subspecies *sativa* is now commonly and legally cultivated in many European countries for the production of cannabidiol/cannabidiolic acid (CBDs) - the most abundant non-psychoactive cannabinoids - as well as seeds which represent an

excellent source of nutrients such as lipids, proteins, carbohydrates, minerals, vitamins, amino acids and essential fatty acids as well as insoluble fiber; edible oil and flour for human use can be obtained from *C. sativa* [11]. It has been shown that even the sprouts obtained from the germination of *C. sativa* seeds represent an interesting example of functional food because sprouts of three/five days are rich in compounds with anti-inflammatory activity such as the prenylflavonoids, cannaflavins A and B [9].

Interestingly, in the '70s of the last century, Slatkin et al. (1971) [12] and Sethi et al., (1977) [13] demonstrated the presence of triterpenoids and sterols in the root extracts of mature *C. sativa* plants. More recently, Jin Dan et al. (2020) [14] have completely profiled the groups of secondary metabolites in the individual parts of the plant, highlighting the presence of sterols (ST, stigmasterol, campesterol) and triterpenoids (FR and EFR) in mature *C. sativa* roots, paving the way to the reappraisal and implementation of the therapeutic potential of *C. sativa* in all its parts.

Although historically *C. sativa* roots were widely used as medicines to treat inflammatory conditions, joint pain, gout and more, the therapeutic potential of *C. sativa* roots has been largely ignored in modern times. Indeed, few studies have examined the composition of *C. sativa* roots and their medical potential [15] , and even less are the studies in which alternative methods are considered for the production of *C. sativa* roots or to increase their content of biological active molecules.

The relative lack of similar studies likely depends on the fact that the research on *C. sativa* is mainly focused on the most widespread cannabinoids that are not significantly present in the roots [15] .

With the aim of filling this gap, in the present work *C. sativa* var. Kompolti - a legal variety routinely used also for food production purposes - has been cultivated through aeroponics [16], a method allowing the plants to grow in a highly controlled manner, free of contaminants and suspended in an environment devoid of soil or other support means. This system has been

selected among others as it theoretically allows a greater production of roots as compared to traditional cultivation in soil, with the additional advantage that there is no need for time consuming and expensive rinsing procedures to isolate and process the roots. (Figure I. 2.)

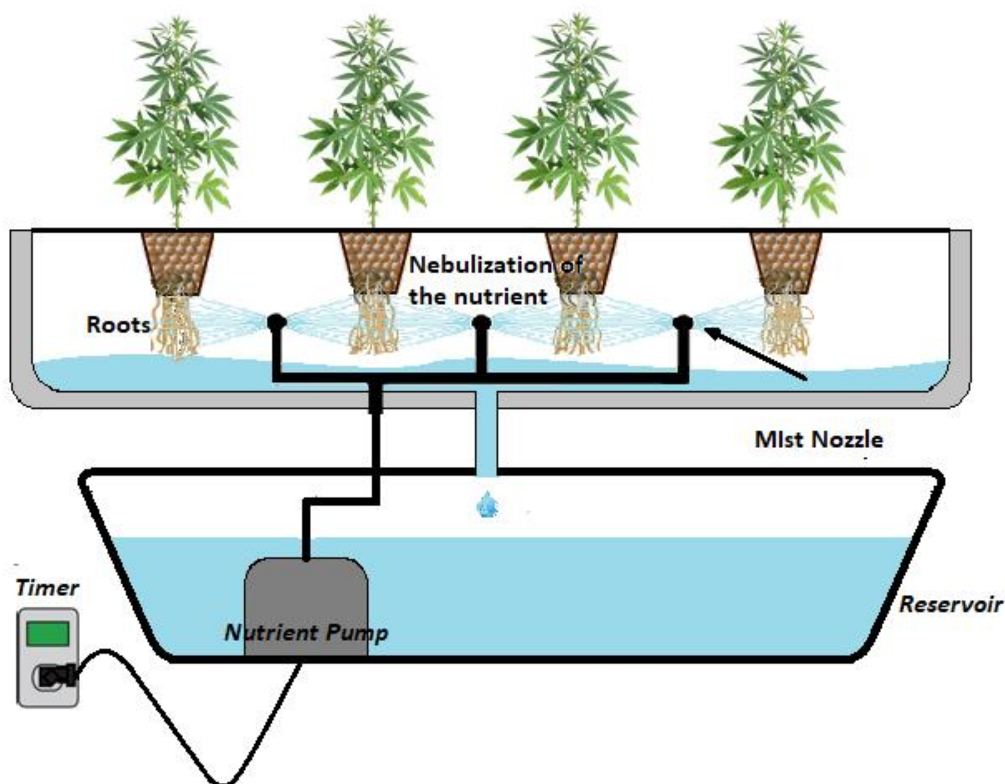


Figure I.2. Aeroponic system.

Therefore, the aeroponics culture should provide a greater production of *C. sativa* roots secondary bioactive metabolites.

In the first part of this thesis the morphological features of plants grown in aeroponics (AP) or in soil (SP) have been studied and compared, and the composition of roots has been analyzed, with the aim of determining the yield of relevant compounds which could be included as active ingredients in the formulation of health promoting and health care products.

In the second part of this research work, the antioxidant and anti-inflammatory properties of the ethanolic extract of *C. sativa* aeroponic roots (APEX) and of its major compounds have been evaluated.

Chapter 1

Yield, Characterization, and Possible Exploitation of *Cannabis sativa* L. Roots Grown under Aeroponics Cultivation.

1.1 Materials and methods

1.1.1 Chemicals and reagents

Extraction solvents (analytical grade), cholesterol, ST, stigmasterol, campesterol, FR, EFR and saturated *n*-alkanes standard (C7–C40) were obtained from Sigma-Aldrich (St. Louis, MO).

1.1.2. Plant material and cultures

C. sativa Kompolti seeds were supplied by Appennino Farm, Gaggio Montano, Bologna (Italy), lot B30756201900001. The seeds were germinated in filter paper wetted with distilled water - 10 seeds in glass Petri dish 14 cm diameter - in the dark, at a constant temperature of 25 °C. After 4 days the rooted seeds were transferred into plastic pots, with a diameter of 5.5 cm and a height of 6.0 cm containing a mixture of 50% peat and 50% vermiculite wetted with Hoagland's half strength nutrient solution up to when the seedlings were not ready for transplanting. The pots (one rooted seed per pot) were placed in a climatic cell with a photoperiod of 18 hours (lamp and conditions as below) until the first two true leaves are fully developed. At this point, five *C. sativa* seedlings of uniform size, length of the first two true leaves equal to about 3.0 cm, were selected each time. The base of the stem of each seedling was fixed with a sponge, placed in a reticulated pot for aeroponics (diameter 6.0 cm and height 6.0 cm) and this placed on the hole of the lid of a tub for aeroponic cultivation in black PVC (50.0 cm x 50.0 cm x 34.0 cm height) capable of hosting five plants each time at a distance of fifteen cm from each other. Complete Haogland nutrient solution was sprayed at

the roots of the plants for a duration of fifteen minutes every hour, and recovered via a closed circuit. Electrical Conductivity (EC) and pH of the nutrient solution were controlled at 0.6-0.7 ms/cm and 6.0, respectively. The aeroponics culture system was maintained in a climatic cell with photoperiod as above with high pressure sodium lamps (Sonlight AGRO 250W grown+bloom). Photosynthetic photon flux density (PPFD) at the plant canopy was about 150 $\mu mol/m^2 \bullet s$. Temperatures during the periods of light and dark were maintained at 27 ± 1 °C and 22 ± 1 °C, respectively. Relative humidity was 65 ± 5% and mean CO_2 concentration was 670 ± 30 µmol/mol.

For the traditional cultivation in pots, the seedlings with two real leaves (about 3 cm long) were placed in a plastic pot (diameter 30.0 cm and height 30.0 cm), containing a mixture as above (50% vermiculite and 50% peat), 3 plants for pot, spaced 15 cm from each other and watered periodically with the whole Hoagland nutrient solution. The pots were placed in a climatic cell with the conditions described for aeroponics and at the same time as this.

Three culture systems of *C. sativa* Kompolti have been set up: SP (Soil Plant) carried out only for the vegetative phase; AP (Aeroponic Plant) carried out only for the vegetative phase; AEP (Aeroponic elicited Plant) carried out only for the vegetative phase supplemented with the elicitor technique with salicylic acid.

AEP were obtained by adding salicylic (25 µM final concentration) acid to the nutrient solution of one-week old plants, according to Ze-Bo Liu et al. [17].

1.1.3. Biomass production

Five plants from each culture system were harvested three times after 8 weeks of culture. It should be noted that the plants were kept in vegetative phase maintaining the photoperiod constant (18 h). Under these conditions plants could not flower because flowering requires a gradual reduction of the photoperiod (from 18 to 12 h). The root systems were carefully washed with tap water and dried and with absorbent paper to remove excess water and their fresh weights immediately recorded. The other parameters taken into consideration were: dry

weight of the roots (g); fresh and dry weight of the aerial parts (g); height of the aerial parts (cm); diameter of the stems (mm) and surface of the collected leaves at half height (cm).

1.1.4. Extraction of dry C. sativa roots

Powdered *C. sativa* roots (300.0 mg) and 100 μL of the IS solution (cholesterol, 1.128 mg/mL in ethyl acetate) was extracted in ethyl acetate (40 mL) under magnetic stirring for 1.5 h at room temperature, followed by centrifugation at 5000 rpm for 8 min. The supernatant was collected in a flask and the residue was extracted once again in the same manner. The collected organic phases were washed with water (2 x 10 mL) and brine (10 mL) then dried (Na_2SO_4 anhydrous), filtered, and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in 10 mL of ethyl acetate and kept at 4 °C until GC-MS and GC-FID analyses.

1.1.5. Gas Chromatography (GC-MS, GC-FID)

GC-MS analyses were carried out using a Trace GC Ultra gas chromatograph coupled to an ion-trap mass spectrometer (ITMS) detector Polaris Q (Thermo Fisher Scientific, Italy) and equipped with a split-splitless injector. The column was a 30 m x 0.25 mm i.d., 0.1 μm film thickness, fused silica SLB-5ms (Supelco, Sigma-Aldich, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 5 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 7 min. Samples (1 μL) were injected in the split (1:10) mode. Injector, transfer line and ion source were set at 280, 280 and 200 °C, respectively. Helium was used as carrier gas at a flow of 1 mL min^{-1}. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from *m/z* 50 to 650 and a scan rate of 0.8 scan/sec. Identification of metabolites was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library.

A Fisons GC 8000 series gas chromatograph, equipped with a flame ionization detector and a split/splitless injector (Fisons Instruments, Milan, Italy), was used for the quantitation of secondary metabolites. The separation was carried out with a fused silica capillary column DB-5MS UI 30m x 0.250mm x 0.25μm film thickness (Agilent, J&W, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 10 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 15 min. Samples (1 μL) were injected in the split (1:10) mode. Injector and detector were set at 280 °C. Hydrogen was used as carrier gas at a flow of 1.8 mL/min. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Italy).

Quantification of the analytes in the dry *C. sativa* roots was performed using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements. When standards were unavailable, the quantification of the target analyte was carried out using the relative response factor of available standards of similar chemical structure.

The retention indices (RIs) were calculated by comparing the retention time of each compound with those of a homologous series of *n*-alkanes standard (C7-C40) under the same chromatographic conditions.

1.1.6. Statistics

Stastical analyses were carried out to compare each root metabolite quantified in AP AEP and SP using Tukey's multiple comparison tests.

1.2 Results

1.2.1. Biomass production

The growth of *C. sativa* var. Kompolti plants used in this study was significantly influenced by the two systems tested - i.e. conventional *vs* aeroponic - with aeroponics promoting a significantly more rapid and intense growth of both aerial parts and root systems (Figure 1.1; Table 1.1). On average, after eight weeks

of parallel cultivation, the roots of APs showed a 64 and 13 folds higher fresh (FW) and dry weight (DW) as compared to SP, respectively; the aerial parts showed a 39 and 44 folds higher FW and DW; the stems' average diameter and the mean leaves area increased by 3.89 folds and 8.9 folds, respectively. AP and AEP reached an almost double height (ca. 70 cm) as compared to SP (ca. 30 cm). Finally, the addition of the elicitor salicylic acid to the nutrient sprayed did not result in any significant variation of these parameters in AEP as compared to AP (Table 1.1, Figure 1.1).

Table 1.1. morphological features of different plant

Culture system	Height (cm)	Roots weight FW/DW (g)	Aerial parts weight FW/DW (g)	Stem average diameter (mm)	Leaves average area (cm^2)
SP	32.6±0.7	3.7±0.4/1.2±0.1	15.8±0.7/2.9±0.4	2.13±0.1	5.2±0.4
AP	70.51.8	238.7±4.1/15.8±0.5	616.9±3.0/129.3±0.7	8.30±0.5	48.3±3.7
AEP	72.5±1.3	246.1±4.3/16.7±0.4	656.3±3.1/137.1±1.6	8.21±0.4	49.4±3.4

Abbreviations: FW = Fresh weight; DW = dried weight; SP = Soil Plant; AP = Aereoponic Plant ; AEP = Aereoponic Elicitated Plant

Figure 1.1. C. sativa L. plants and roots cultured either under soil (A; C) or aeroponic conditions (B; D).

1.2.2. Extract characterization[1]

The content of the main roots' bioactive constituents has been comparatively determined in SP, AP and AEP plants by GC-MS. The main compounds identified were the phytosterols campesterol, stigmasterol and ST and the triterpenes EFR and FR (Figure 1.2). Figure 1.3. shows a typical gas chromatogram of the ethyl acetate extract of powdered *C. sativa* roots. On a per

[1] Unfortunately, due to logistic problems in analyzing the inflorescences, the determination of CBD/THC ratio in AP *vs* SP is still underway. As a consequence, the results could not be included in this thesis.

DW basis (Table 1.2; Figure 1.4) the amount of bioactives is higher in SP as compared to both AP and AEP. As to the single constituents, the amount of EFR and FR was far higher in SP, that of campesterol and stigmasterol was similar in the three types of cultures while ST was higher in AP and AEP. On a percent basis (Table 1.2 and Figure 1.5), FR and EFR were the most expressed compounds in SP, while ST in AP and AEP; finally, the amount of ST and EFR in AEP as compared to AP decreased and increased, respectively.

Table 1.2. Content of the main bioactive compounds present in 100.0 mg of dry SP, AP, and AEP roots

	Culture system						
	RI	**SP**		**AP**		**AEP**	
Compound		**(μg)[a]**	**%**	**(μg)[a]**	**%**	**(μg)[a]**	**%**
Campesterol	3179	17.8±0.6	4.8	17.2±0.	10.9	18.5±0.2	10.0
Stigmasterol	3205	26.1±0.2	7.0	20.2±0.	12.7	30.1±1.0	16.3
β-Sitosterol	3263	40.4±0.0	10.8	68.7±1.	43.3	59.0±7.8	32.0
Epi-friedelanol	3433	92.2±0.5	24.6	23.9±0.	15.0	42.8±1.5	23.2
Friedelin	3448	197.5±1.3	52.8	28.8±1.	18.1	33.9±1.0	18.5
Total		374.0±2.6		158.8±1		184.3±8.5	

Abbreviations: RI = Retention Index, SP = Soil Plant, AP = Aeroponic Plant; AEP = Aeroponic Elicitated Plant ; [a]Data are expressed as the mean value ± standard deviation; n = 3 repetitions.

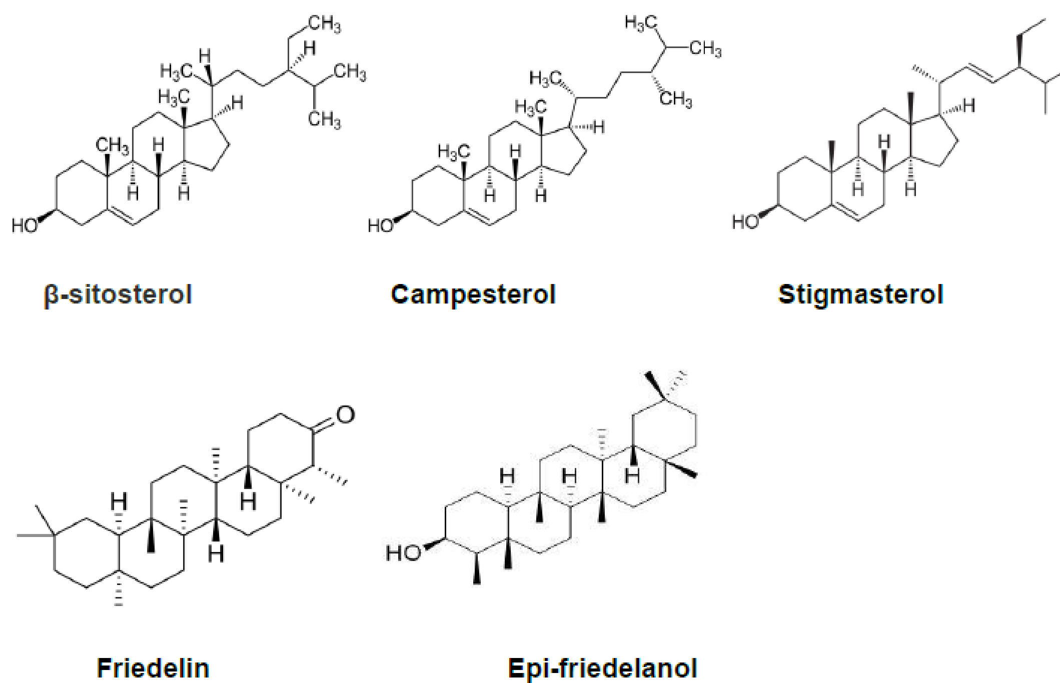


Figure 1.2. Chemical structure of the main chemical compounds identified by GC-MS in Aeroponic Plant (AP) Aeroponic Elicited Plant (AEP) and Soil-cultivated Plant (SP) root extracts.

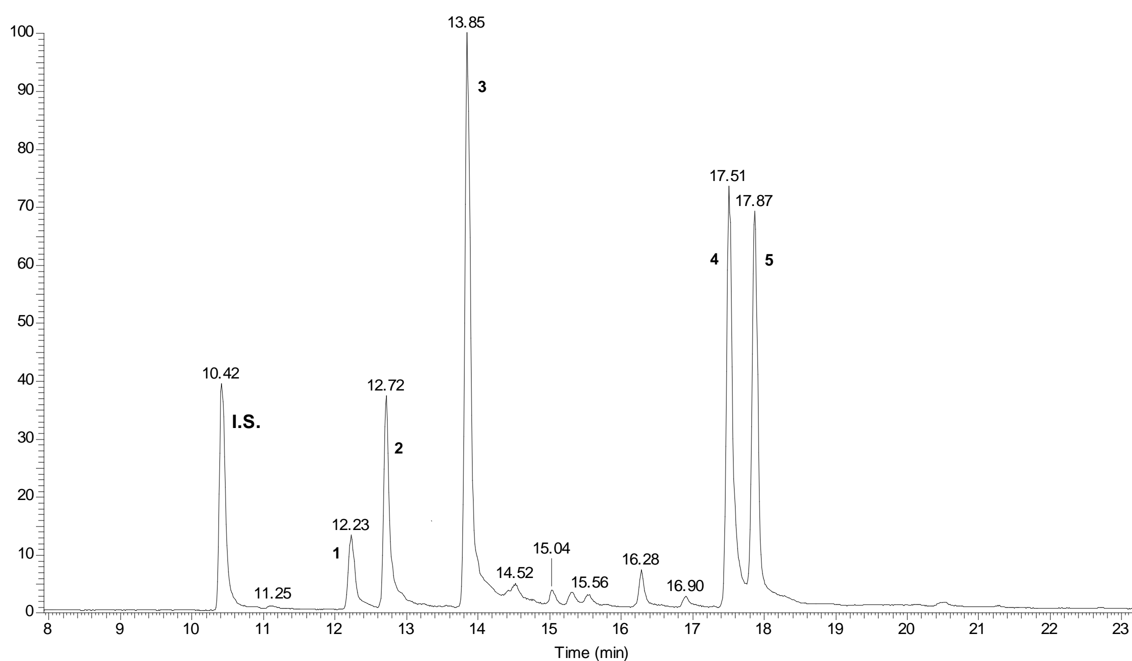


Figure 1.3. Typical total ion current (TIC) chromatogram obtained from the ethyl acetate extract of powdered *C. sativa* roots. Peaks: **I.S.** (internal standard, cholesterol), **1** (campesterol), **2** (stigmasterol), **3** (ST), **4** (EFR), **5** (FR).

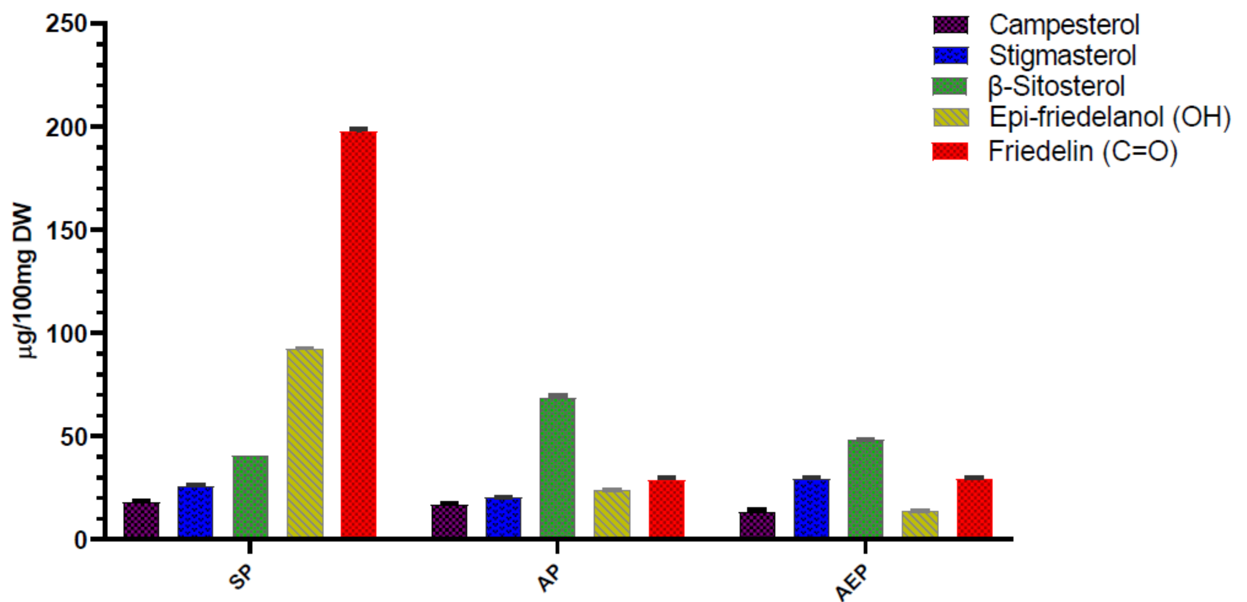


Figure 1.4. Concentration of the main bioactive compounds of Aeroponic Plant (AP) Aeroponic Elicited Plant (AEP) and Soil-cultivated Plant (SP) root extracts of *C. sativa* L. Data are expressed as µg/100mg DW ± SEM.

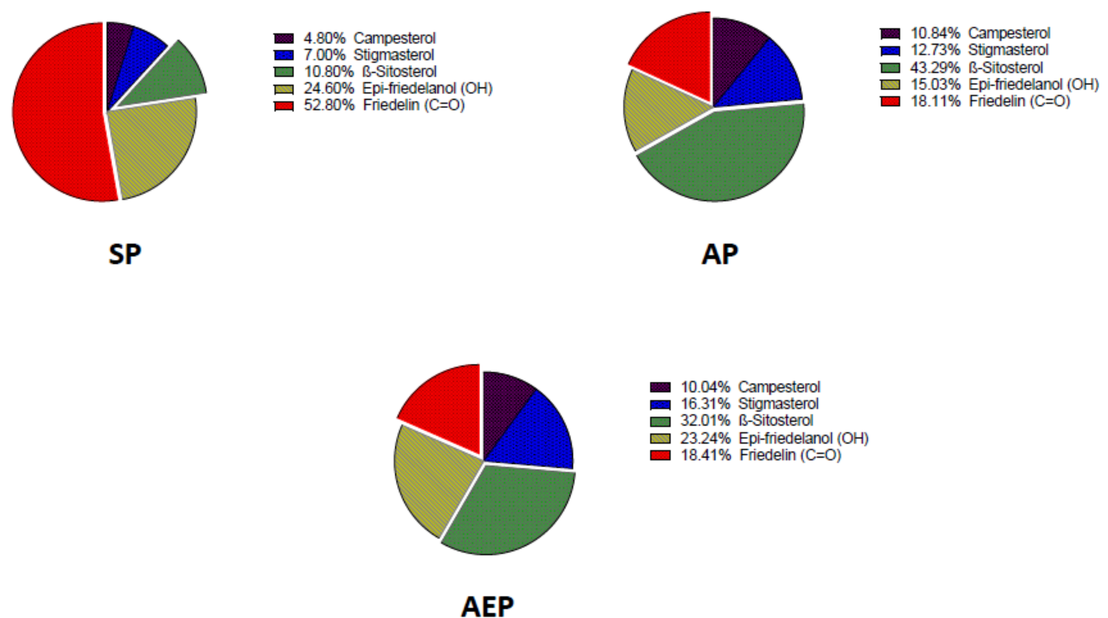


Figure 1.5. Percentage of the main bioactive compounds of the *C. sativa* L. root extracts of Aeroponic Plant (AP) Aeroponic Elicited Plant (AEP) and Soil-cultivated Plant (SP).

Notably, when analyzed on a "per plant" basis the results were markedly different (Figure 1.6). Indeed, since the biomasses of the plants grown in aeroponics were heavier (13 to 64 folds, roots DW and FW, respectively) both AP and AEP contained significantly higher root bioactives' amounts (Table 1.3; Figure 1.6). The amounts of ST from AP (10,86 ± 0,72 mg) and AEP (9,89 ± 2,17 mg) roots were, respectively, 23 and 20 times higher than in SP (0,49 ± 0,05 mg); FR, whose concentration on a per weight basis was significantly higher in SP, turns to higher per plant values in AEP and AP (4,55 ± 0,47 mg; 5,67 ± 0,4mg), respectively than SP (2,37 ± 0,3mg) (Table 1.3; Figure 1.6). Similar proportions have been observed for campesterol, stigmasterol and EFR (Table 1.3).

Table 1.3. Total amount of chemical compounds in SP, AP and AEP cultivation.

Culture system	SP	AP	AEP
	Total mg/g DW plant	**Total mg/g DW plant**	**Total mg/g DW plant**
Campesterol	0,21 ± 0,03 mg	2,72 ± 0,2mg	3,09 ± 0,14 mg
Stigmasterol	0,31 ± 0,04 mg	3,19 ± 0,17 mg	5,03 ± 0,4 mg
β-Sitosterol	0,49 ± 0,05mg	10,86 ± 0,72 mg	9,9 ± 2,17 mg
Epi-friedelanol (OH)	1,11 ± 0,14mg	3,77 ± 0,17 mg	7,16 ± 0,6 mg
Friedelin (C=O)	2,37 ± 0,3mg	4,55 ± 0,47 mg	5,67 ± 0,4mg

Abbreviations: FW = Fresh weight; DW = dried weight; SP = Soil Plant ; AP = Aereoponic Plant ; AEP = Aereoponic Elicitated Plant

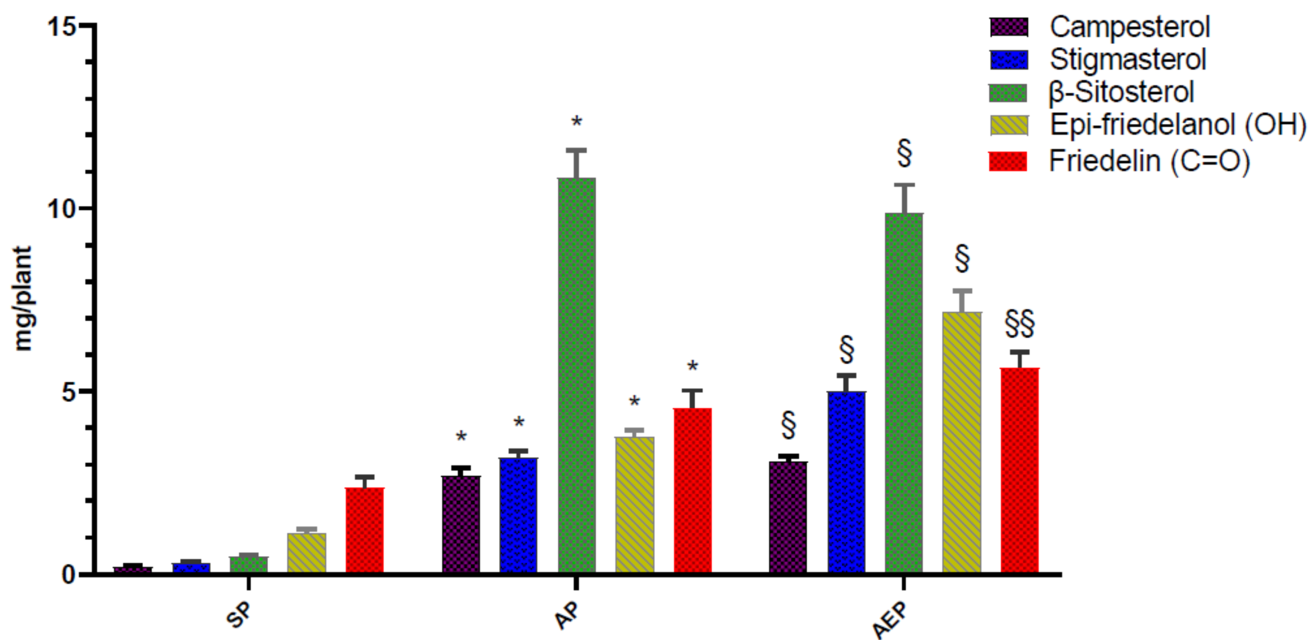


Figure 1.6. Total amount of the main bioactive compounds of Aeroponic Plant (AP) Aeroponic Elicited Plant (AEP) and Soil-cultivated Plant (SP) *C. sativa* roots. Data are expressed as mg per plant. * $p < .0001$ AP *vs* SP; §§ $p < .0001$ and § $p < .05$ AEP *vs* AP (Tukey’s multiple comparisons test).

1.3. Discussion

This study was undertaken for the reason that *C. sativa* roots - although potentially valuable - are a neglected source of bioactive compounds. The fact that roots lack the most pharmacologically-relevant compounds - namely Δ^9-THC and CBDs - and the relative complexity of root processing probably account for this scarce interest for *C. sativa* roots. However, unlike SP, roots harvesting/processing from AP is far easier, cheaper and flexible. Indeed AP roots are clean and free from the parasites and contaminants normally present in the soil; more interestingly, as shown in the present study and elsewhere [18,19] , the absolute and relative yield of bioactives can be modulated simply varying the composition of the nutrient sprayed onto the roots by adding specific elicitors. Moreover, roots of AP may meet the standards of organic cultivations, which today represent a desired and high-quality end point.

The chemical characterization of SP, AP and AEP roots indicates that 1) the phytosterols ST, campesterol and stigmasterol and the triterpenoids FR and EFR represent the major components of interest; 2) the relative proportion of these constituents was significantly affected by the cultivation system.

Here we demonstrate that aeroponics applied to *C. sativa* var. Kompolti results in a significant modification in the yield of plant biomasses and in the net and relative abundance of root bioactive compounds, as compared to conventional soil cultivation. Indeed, the biomass of AP (both aerial parts and roots) after 8 weeks of cultivation strikingly outpaces that of SP. In particular, the overgrowth of roots was impressive with a 64 and 13-fold increase in FW and DW, respectively, as compared to the roots from SP. The greater increase in FW is likely due to the very high hydration rate attainable under aeroponics.

This overgrowth has been found to occur also in other plants: Li et al.[20]; showed that roots' DW of two varieties of lettuce grown in aeroponics, was significantly higher than that obtained not only cultivating plants in soil, but also with the hydroponic technique.

Other authors [21] showed that aeroponics of *Crocus sativus* (saffron) promoted a more robust growth of the root system which, however, was not paralleled by a proportional overgrowth of the aerial parts. This observation suggests that, under aeroponics a larger root system does not necessarily result in a correspondingly greater biomass of the aerial parts. We also found that, although both roots and aerial parts of AP were invariably greater and heavier than SP, aerial parts and roots grew to different extents. The differential overgrowth observed here and elsewhere may be the expression of a plant type-dependent effect of aeroponic cultivation. In fact, both the traditional cultivation method on substrate and the hydroponic one involve a continuous immersion of the roots in nutrients and water. In some cases, this can stimulate more efficiently the growth of the aerial part of the plant as compared to the aeroponics method, where the roots are suspended in a chamber, wetted at regular intervals with the nutrient solution and with virtually unlimited access to air O_2. The extensive oxygen availability likely represents the most important advantage of the aeroponic culture method over conventional and hydroponic ones [22].

On a pharmaceutical perspective, Hayden et al. [23,24] have shown that aeroponics can represent an excellent system for the production of roots from medicinal plants in which these organs are used for the extraction of active molecules such as, for example, burdock (*Arctium lappa*) and ginger (*Zingiber officinale*). Importantly, as compared to other techniques aeroponics allows to obtain perfectly clean root apparatuses which can be immediately harvested, extracted, lyophilized, micronized or subjected to other processing methods. A further advantage of aeroponics is that the culture medium can be easily and precisely enriched with specific elicitors which can further enhance the bioactives' yield.

Although more appreciated in the market for its content in CBDs from plant's inflorescences and for the wide utilization of the aerial parts, here we applied and tested aeroponics to *C. sativa,* whose roots exhibit a fairly high content in potentially valuable constituents characterized by pharmacological,

nutraceutical and cosmeceutical attractive activities. Notably, we show that the yield of these components on a per plant basis was invariably and significantly higher in AP and AEP as compared to SP, and that using salicylate as elicitor [25,26] accumulation of all bioactives could be attained (with the exception of ST which only slightly decreased as compared to AP).

Hence these findings could pave the way for a rational implementation of *C. sativa* aeroponic cultivation based on the development of mixtures of specific elicitors to increase and optimize the yield of root bioactive constituents. In this light elicitation may allow to obtain different *C. sativa* roots' phytocomplexes with specific attitudes resulting from the relative proportion and peculiar biological features of each component.

As briefly anticipated above, the properties of the bioactives identified in *C. sativa* roots largely justify their use - either singularly or as phytocomplex - for the preparation of health promoting products. The most abundant components are represented by phytosterols, particularly ST. ST is a sterol found in almost all plants. It is one of the main subcomponents of a group of plant sterols known as phytosterols that are very similar in composition to cholesterol. High levels are found in rice bran, wheat germ, corn oil, and soybeans; peanuts and their products such as peanut oil, peanut butter, and peanut flour; *Serenoa repens,* avocados, pumpkin seed, *Pygeum africanum,* and cashew fruit.

The antihypercholesterolemic effect of ST has been reported by Cicero et al. [27]; thirty-six human volunteers took 2 g/day of ST and 8 g/day of soy protein for forty days and after this period they showed a significant decrease of LDL (low-density lipoprotein), VLDL (very low density lipoprotein), TG (triglycerides) levels and a significant increase of HDL (high-density lipoprotein) [27].

Studies have suggested that ST inhibited proliferation of human prostate cancer cells [27] and growth of tumors derived from PC-3 human prostate cancer cells [28,29].

The ability of ST and other phytosterols to inhibit aromatase and 5-alpha-reductase has been well documented, and this inhibitory capacity has been

exploited to treat pathologies such as benign prostatic hyperplasia and androgenetic alopecia [30–32]. One of the richest sources of phytosterols, particularly ST, is *Serenoa repens* whose extracts have been largely studied and used in nutraceutical formulations (Permixon, Calprost, Difaprost) proposed for the adjunct therapy of benign prostatic hyperplasia [29,33,34].

The concentration of ST in *S. repens* extract, 0.454 ± 0.018 mg/g, dry mass [35], is a hundred times lower than those we found in *C. sativa* extracts; such a finding would make the use of *C. sativa* roots extracts for nutraceutical applications as plausible as those from *S. Repens*. As compared to other sources, *C. sativa* roots have very low content in lipids, proteins and carbohydrates: hence, they could be taken by overweight/obese, diabetic or hypercolestolemic patients without increasing their caloric intake.

FR, a pentacyclic triterpenoid which can be found in many plants, displays a wide spectrum of anti-inflammatory, antipyretic, anticarcinogenic and antitumor effects with low toxicity [36].

FR was reported to promote apoptosis and inhibit the growth of various tumor cell lines including MCF-7 human breast cancer and AML-196 human leukemia cells [37–40].

FR also possesses other remarkable properties such as: *in vivo* anti-inflammatory, analgesic and antipyretic effects in adult Wistar rats [41] membranes stabilization [42], hypoglycemic effect in diabetic rats [43] gastroprotective and antiulcerogenic activity [44], estrogenic activity [45], antihyperlipidemic and antihypertensive effect [46].

FR shows remarkable antioxidant capacity, comparable to that of BHT or ascorbate [47].

Finally, FR, thanks to its antimycobacterial activity, has been proposed as a natural antituberculosis agent [48]. Interestingly, this usage parallels that of *C. sativa* leaf macerated in warm water and taken as a treatment for tuberculosis by the Bapedi healers of Limpopo Province, South Africa [49].

Another molecule present in significant percentages is EFR (Figure 1.2; Figure 1.3). This compound is another pentacyclic triterpenoid that shares a large part of the molecular structure with FR, with the difference that this latter has a cyclohexanone, while EFR a cyclohexanol.

EFR is present in several plants, such as in the root barks of *Ulmus Davidiana* [50], *Cayratia trifolia*[51–53], *Vitis trifolia*[54], *Celtis sinensis* [55], *Mallotus apelta* [56]and *Ulmus pumila* [57]. This triterpenoid has been reported to have anticancer [54,58], anti-inflammatory [55] and anti-senescence activity [50]. In particular, Yang et al. [50] who found that EFR suppresses cellular and replicative senescence in human fibroblasts and human umbilical vein endothelial cells, proposed its use in the formulation of nutraceuticals or cosmeceutical aimed at modulating tissue aging or aging-associated diseases.

Chapter 2

Characterization of the biological activity of *Cannabis sativa L.* roots grown under aeroponic system

2.1 Materials and methods

2.1.1. Ethanolic extract of aereponic plant roots (APEX)

The *C. sativa*, roots were collected after eight weeks of cultivation in the aeroponic system. Two to 5 g of roots were chopped with scissors and subsequently ground by pestle and mortar, and finally 100 ml of ethanol/water (80:20) were added.

Everything was placed in an Erlenmeyer flask and subjected to a 24-hour magnetic agitation. At the end, the final product was aliquoted in centrifuge tubes and dried using a Savant concentrator (Thermo Scientific, San Jose, CA, USA).

We used a cold-spun to avoid degradation of the thermolabile compounds contained in the *C. sativa* roots. After about 6 h the dry extract has been weighed obtaining from 9 to 10 mg of dry weight per tube.

The dried samples were stored at 4 °C until use. For experiments, the dried APEX was resuspended in DMSO.

2.1.2. Cell culture

Human promonocytic U937 cells were cultured in suspension in RPMI 1640 medium (Corning, New York, USA) supplemented with antibiotics (100U/ml penicillin, 100 μg/ml streptomycin), 1.2 mM glutamine, and 10 % fetal bovine serum, at 37 °C in a humidified atmosphere of 5% CO_2.

The human articular chondrocyte cell line HC-a (Sciencell, Carlsbad, CA, USA) was maintained in DMEM (Corning, New York, USA) supplemented with

antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 1.2 mM glutamine, and 10 % fetal bovine serum, at 37 °C in a humidified atmosphere of 5% CO_2.
HC-a cultures were passaged on, reaching 80% confluence, using trypsin–EDTA solution (Sigma-Aldrich, St. Louis, MO, USA). The experiments were performed in the third culture passage (P3).

2.1.3. DPPH radical scavenging activity

The DPPH free radical scavenging assay was performed as reported in Sestili et al. [59] The decrease in absorbance (517 nm) was recorded after 10 min at room temperature in the dark.
The scavenger effect was calculated as % = [(A517 nm of blank- A517 nm of samples /A517 nm of blank] x100.
The EC_{50} values were calculated by linear regression from the dose/response plots and represent the concentration required to provide 50% free radical scavenging activity.

2.1.4. Chelating effect on Fe^{2+}

The chelating effect on Fe^{2+} was measured as previously reported [60]. 200 μl of APEX, FR, ST and EFR were mixed with 740 μl of deionized water; the mixture was reacted with 20 μl $FeSO_4$ (2 mM) and 40 μl ferrozine (5 mM) for 10 min and the absorbance at 562 nm was then spectrophotometrically determined.
Chelating activity was calculated as % = [(A562 nm of blank - A562 nm of sample)/A562 nm of blank] × 100.
EC_{50} represent values represent the concentrations at which ferrous ions are chelated by 50%.

2.1.5. Cell treatments

In order to evaluate the antioxidant activity, stock solutions of H_2O_2 were freshly prepared in distilled water and diluted to the proper concentrations. APEX, FR, ST and EFR were added to cultures 24 h before addition of the oxidant; oxidative

treatments were performed in 1 mL of medium in 6-well TC plate, containing 4×10^{5} cells/treatment condition by adding 100 μM H_2O_2 for 1 h. Cells were washed with 2 ml of saline A (8.182 g l−1NaCl, 0.372 g l−1KCl, 0.336 g l−1$NaHCO_3$ and 0.9 g l−1glucose) and analyzed either immediately or following further 24 or 48 h post-challenge growth in fresh, drug-free medium.

To evaluate the anti-inflammatory properties of APEX, FR, ST and EFR, U937 cells (1×10^{6} cells/ml) were stimulated for 12 h with 1 μg/ml of lipopolysaccharide (LPS; Sigma -Aldrich). APEX, FR, ST and EFR were added 24 h before LPS stimulation.

At the end of the 12 h LPS stimulus the cells were assayed for inflammatory markers gene expression analysis. In other experiments, after exposure to LPS, cells were resuspended in fresh medium and grown for further 24h. At the end of this stage, the medium was collected to quantify the IL-6 protein level with ELISA assay.

2.1.6. Cell viability assay

Cell number and viability were determined the trypan blue exclusion assay. Briefly, an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a LUNA-II™ Automated Cell Counter (Logos biosystem, Dongan-gu Anyang-si, South Korea). Results are expressed as the percent ratio between the number of viable cells in treated sample and that in sham-treated samples [59].

2.1.7. Fast Halo Assay (FHA)

Immediately after the treatments, the cells were resuspended at 4.0×10^{4} /100 ml in ice-cold phosphate-buffered saline (8 g l−1 NaCl, 1.15 g l−1 Na_2HPO_4, 0.2 g l−1 KH_2PO_4, 0.2 g l−1 KCl) containing 5 mM EDTA: this cell suspension was diluted with an equal volume of 2.0% low-melting agarose in phosphate buffered saline and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling on ice, the coverslips were removed and the slides were

immersed in NaOH 0.3 M for 15 min at room temperature. Ethidium bromide was directly added to NaOH during the last 5 min of incubation. The slides were then washed and destained for 5 min in distilled water. The ethidium bromide-labelled DNA was visualized using a Leica DMLB/DFC300F fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and the resulting images were digitally recorded on a personal computer and processed with an image analysis software (Scion Image). The extent of strand scission has been quantified by calculating the nuclear diffusion factor (NDF), which represents the ratio between the total area of the halo plus nucleus and that of the nucleus [61].

2.1.8. Expression of inflammatory genes: RNA isolation cDNA synthesis and quantitative Real-Time PCR

Total RNA from treated cells was extracted according to E.Z.N.A. total RNA kit manual (VWR international, Milan, Italy). A E.Z.N.A RNase-Free DNase I set (VWR international, Milan, Italy) digestion step was performed on all the RNA samples before subsequent reactions.

The quantity of RNA was estimated spectrophotometrically at 260 nm (SpectraMax QuickDrop Micro-Volume Spectrophotometer, Molecular Devices, San Jose, CA, USA).

Reverse transcription of cDNA was performed from 500 ng of total RNA using the PrimeScript™ RT Reagent Kit (Takara Bio Europe, Saint-Germain-en-Laye, France).

From mRNA expression analyses, the cDNA products were subjected to Real-Time SYBR-green-based quantitative PCR on a StepOnePlus™ Real Time PCR System (Applied Biosystems, Monza, MB, Italy) in a final volume of 20 ul using Power Up master mix (Life Technologies) and 0.3 μM of the primer pairs reported in Table 2.1.

The relative levels of target mRNA expression were normalized to values obtained for the tata-binding protein (TBP), used as a "housekeeping gene" simultaneously with the experimental samples.

The RT-PCR conditions were: 50° C for 2 min, 95° C for 2 min followed by 40 cycles of two-steps at 95° C for 15 s, 60° C for 60 s and finally three-steps at 95° C for 15 s, 60° C for 60 s and 95° C for 15 s.

For miRNA analysis, human miR-146a were quantified by RT–qPCR using TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. The RT–qPCR data were standardized to RNU44 (reference miRNA).

Product specificity was examined by dissociation curve analysis. Results were calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) and were expressed as fold change related to untreated control (CTRL).

Each sample was tested in triplicate by RT–qPCR.

Table 2.1. Primer sequences for Real-Time PCR

Gene	*Primer*	*Sequence*
IL-6	Forward	AGGGCTCTTCGGCAAATGTA
IL-6	Reverse	GAAGGAATGCCCATTAACAACAA
IRAK-1	Forward	CAGACAGGGAAGGGAAACATTTT
IRAK-1	Reverse	CATGAAACCTGACTTGCTTCTGAA
TNF- α	Forward	CTGCTGCACTTTGGAGTGAT
TNF-α	Reverse	TCTCAGCTCCACGCCATT
IL-8	Forward	GCAGAGGGTTGTGGAGAAGT
IL-8	Reverse	GCTTGAAGTTTCACTGGCATC
IkBα	Forward	GGTGCTGATGTCAATGCTCA
IkBα	Reverse	ACACCAGGTCAGGATTTTGC
iNOS	Forward	CCCTTCAATGGCTGGTACATGG
iNOS	Reverse	ATGTTGATCTCAACGACAGCC

TBP	Forward	TGCACAGGAGCCAAGAGTGA
TBP	Reverse	CACATCACAGCTCCCCACCA

Note: IL-6, interleukin-6; IL-8, interleukin-8; IRAK-1, Interleukin 1 Receptor Associated Kinase 1; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; TNF-α, tumor necrosis factor-alpha; iNOS, Inducible nitric oxide synthase; COX-2, Cyclooxygenase-2; TBP, Tata binding protein.

2.1.9. Enzyme-linked immunosorbent assay (ELISA)

The levels of secreted IL6 in cell culture supernatant were determined using a human IL6 Quantikine ELISA Kit IL-6 in according to manufacturer's instructions.

Supernatant samples were collected after 24 hours recovery time and tested using IL-6 (DY206, R&D Systems, Minneapolis, MN, USA).

2.1.10. In Vitro Wound Healing Assay

HC-a were seeded in 6-well TC plate, incubated at 37 °C and 5% CO_2 and allowed to grow to confluence as monolayers. The monolayers were subjected to a mechanical scratch wound, horizontal along the flask, using the tip of a sterile pipette. Subsequently, the medium of each flask was removed, the cells washed with PBS solution and grown for 24 h with fresh medium containing APEX, FR, ST or EFR. The untreated cells were used as a control. Micrographs of the lesion areas were taken either immediately after scratching (t_0) or after 24h using a phase-contrast microscope (Olympus Ix51 10X objective). To evaluate wound closure, images were analyzed with the ImageJ software to select the total area of the wound region. The percentage of wound closure was calculated using the following equation [62].

$$ (1)\ [(\text{wound area t0} - \text{wound area t})/\text{wound area t0}] \times 100 $$

2.1.11. Statistical Analysis

The statistical analyses were performed using GraphPad Prism version 8.02 for Windows (GraphPad Software).

The variables among treated samples of FHA were compared using One-way ANOVA with Sidak's multiple comparisons test. The differences between samples were considered significant if p-values were < 0.05.

The two-tailed paired Student's t-test was used for the miR-146a, IRAK-1, IL-6 analyses. The results were considered significant at the level of $p < 0.05$. All the experiments were conducted in triplicate. Linear regression analysis was used to calculate the EC_{50} values from DPPH test and Fe^{2+}chelating ability assay.

2.2 Results

2.2.1. Extract characterization

The analysis of root constituents has been determined by GC-MS. The main biologically-relevant compounds identified were the phytosterols ST, campesterol, stigmasterol and the triterpenes EFR and FR (Table 2.2).

Table 2.2. Content of the main bioactive compounds present in aeroponic *C. Sativa* roots.

Compound	Amount [μg/100 mg dry roots][a]
Campesterol	17.2±0.4
Stigmasterol	20.2±0.2
β-Sitosterol	68.7±1.1
Epi-Friedelanol	23.9±0.1
Friedelin	28.8±1.2
Total	158.8±1.9

[a] **Data are expressed as the mean value ± standard deviation; n = 3 repetitions.**

2.2.2. Cytotoxicity and genotoxicity of APEX

The effect caused by continuous exposure to increasing concentrations of APEX on the growth of human promonocytoid U937 cells is shown in Figure 2.1. Concentrations up to 100 μg/ml did not significantly affect cell growth.

Analysis of nuclear DNA in cells exposed to the same range of APEX concentrations shows no significant DNA strand scission (Figure 2.2).

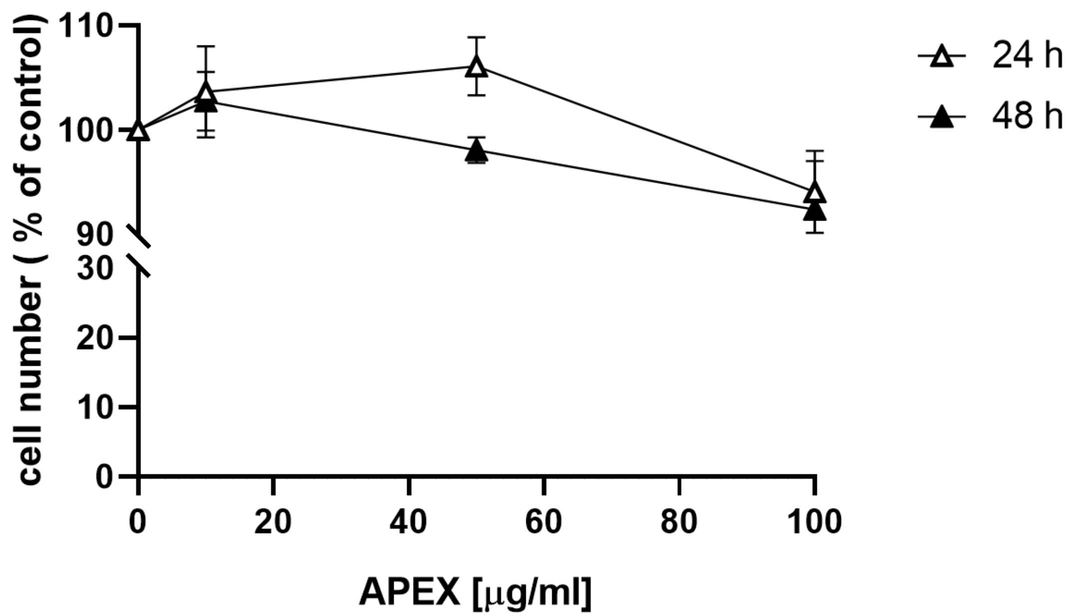


Figure 2.1. Effect of APEX on U937 cell growth. U937 cells were treated with increasing concentrations of APEX for 24 h and grown for further 24 and 48 h in APEX-free medium. The number of viable cells was then determined using the Trypan blue exclusion assay. Values represent the means + SEM of at least three independent determinations.

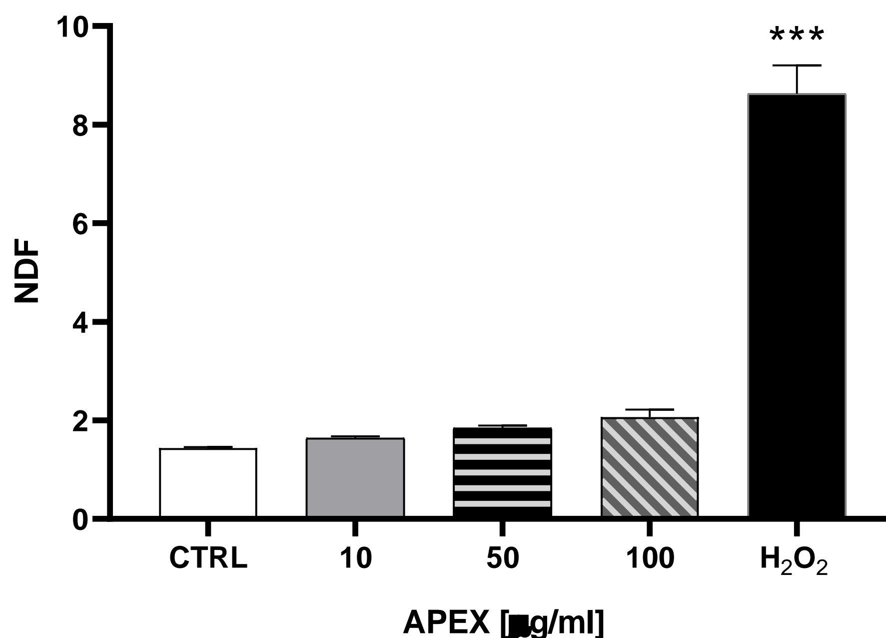


Figure 2.2. Effect of APEX on U937 nuclear DNA. Cells were treated as detailed in Figure 1 and then assayed for DNA damage. The extent of DNA breaks is expressed as NDF (see the methods section). The effect of H_2O_2 (100 μM for 2h) included as a positive control is also shown.

One-way ANOVA- with Dunnett's post hoc test. *** P <0.0001 CTRL vs *H_2O_2* 100 μM.

2.2.3 Antioxidant activity

The antioxidant capacity of APEX - and of its three main components FR, ST and EFR - has been investigated using two different acellular tests, namely the DPPH and the Fe^{2+} chelating ability assays. Concentrations ranging from 10 μg/ml to 1000 μg/ml of APEX showed a remarkable, dose-dependent activity in both DPPH free-radical scavenging effect and Fe^{2+} chelating capacity.

Similarly, the three bioactive components of APEX tested separately were found to exert dose-related scavenging and chelating activities, although to a lesser extent as compared to the whole extract (Figure 2.3).

The EC_{50} values for APEX, FR, ST and EFR calculated from the results of DPPH and Fe^{2+} chelation assays are reported in Table 2.3.

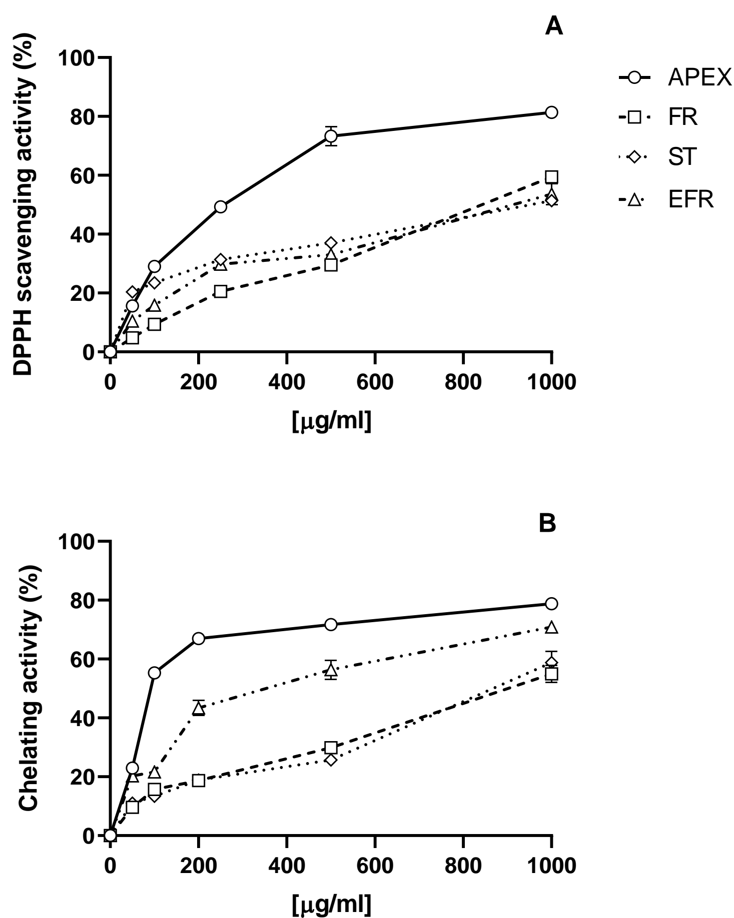


Figure 2.3. In vitro antioxidant capacity. Scavenging effect as assayed with the DPPH test **(A)**; chelating ability on ferrous ions (B). Each value is the mean ± SEM of three independent measurements.

Table 2.3. EC_{50} DPPH and Fe^{2+}chelating activity.

	Antioxidant activity (EC_{50} values, μg/ml)			
	APEX	**friedelin**	**β-sitosterol**	**epi-friedelanol**
DPPH scavenging activity [a]	420.1	832.4	920.2	875.1
Chelating Activity [a]	385.5	883.5	858.8	547.6

(a) Each value represents the mean ± SEM of three independent measurements. EC_{50} values have been calculated from linear regression analysis of the curves depicted in Figure 2.3.

The second set of experiments was aimed to evaluate the antioxidant capacity of APEX in cultured-cell systems. To this end, increasing concentration assessment of APEX on the cytotoxic response of U937 promonocytoid cells exposed to a bolus of H_2O_2 was investigated. The concentrations tested were capable to mitigate oxidative challenge cytotoxicity (Figure 2.4.) in a dose-related fashion. In parallel, the active compounds present in APEX were separately tested under the same conditions; for comparative purposes their concentrations were the same contained in 50 μg/ml APEX. The results obtained (Figure 2.4.) also indicate that the three compounds tested separately behaved similarly to APEX, although none of them was more active than the whole extract.

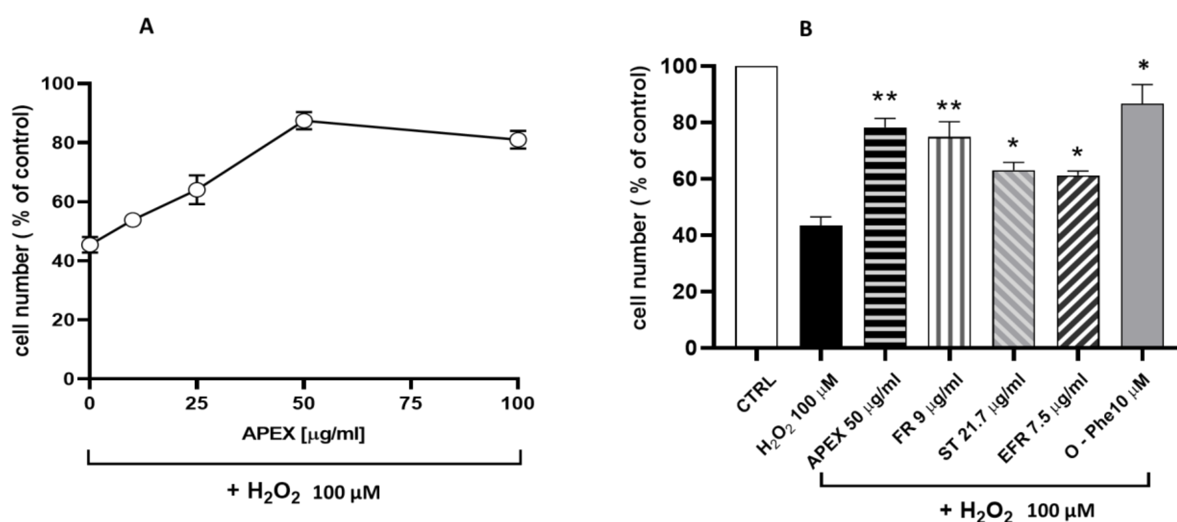
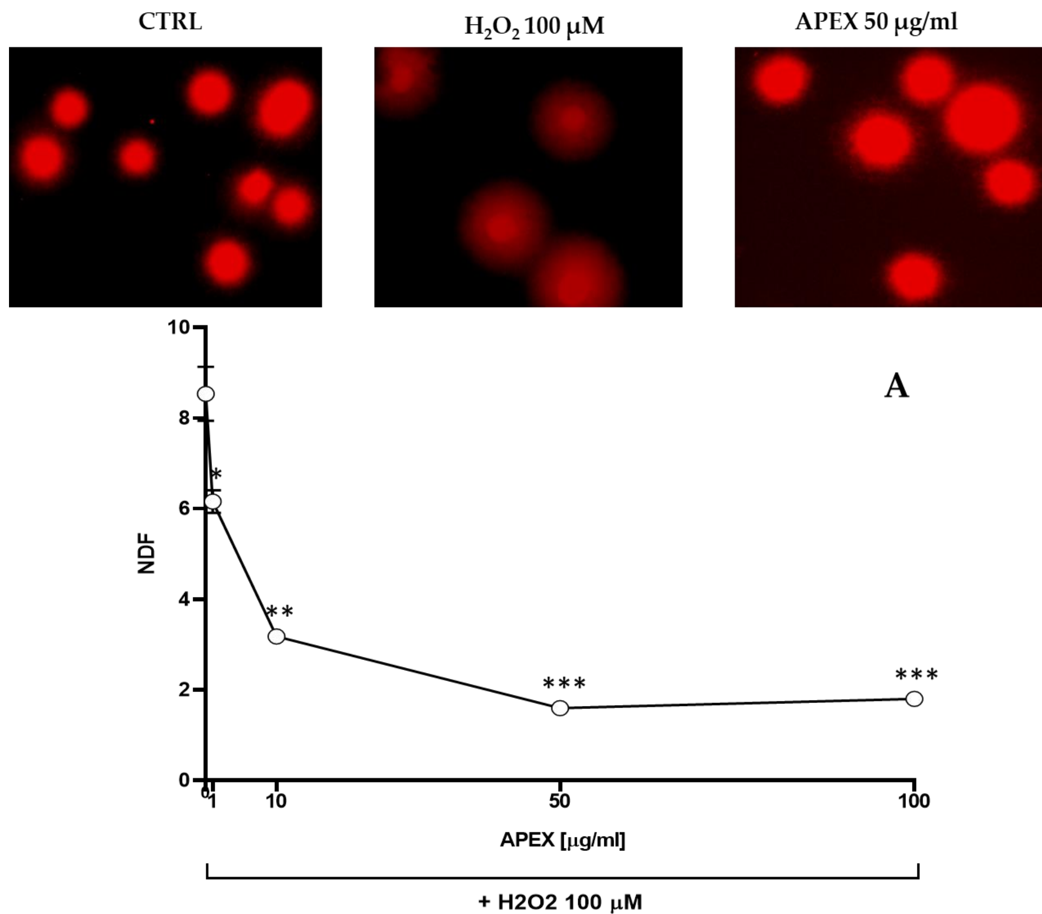


Figure 2.4. Effect of APEX, FR, EFR or ST preincubation on the H_2O_2 cytotoxicity in U937 cells. A) Cells were preincubated for 24 h with increasing concentrations of APEX or B) with APEX (50 μg/ml), FR, EFR or ST (given at the same amounts contained in 50 μg/ml of APEX) and then treated with 100 μM H_2O_2 for 1 h. The viability was determined after 24 h growth in fresh, complete culture medium. Also shown the effect of 30 min pre-treatment with 10 μM of the iron-chelator *o*-phenanthroline (O-Phe) included as positive control. Each point represents the mean ± SEM from four separate determinations, each of which performed in duplicate. * $p < .005$ and ** $p < .005$ (unpaired t-test) compared to control cells.

We have next investigated the ability of APEX, FR, ST and EFR to prevent the oxidant-induced DNA damage in U937 cells [63] (Figure 2.5).

As expected, H_2O_2 challenge (1 h, 100 μM) caused a significant increase in halo size (see the Methods section), which reflects the high accumulation of DNA single strand breaks.

Interestingly, pre-incubation with APEX at three different concentrations dose-dependently prevented DNA strand scission (Figure 2.5.). Again, the three bioactive compounds tested separately were capable of affording genoprotection to an extent similar to APEX (FR and EFR) or slightly, but significantly lower (ST).



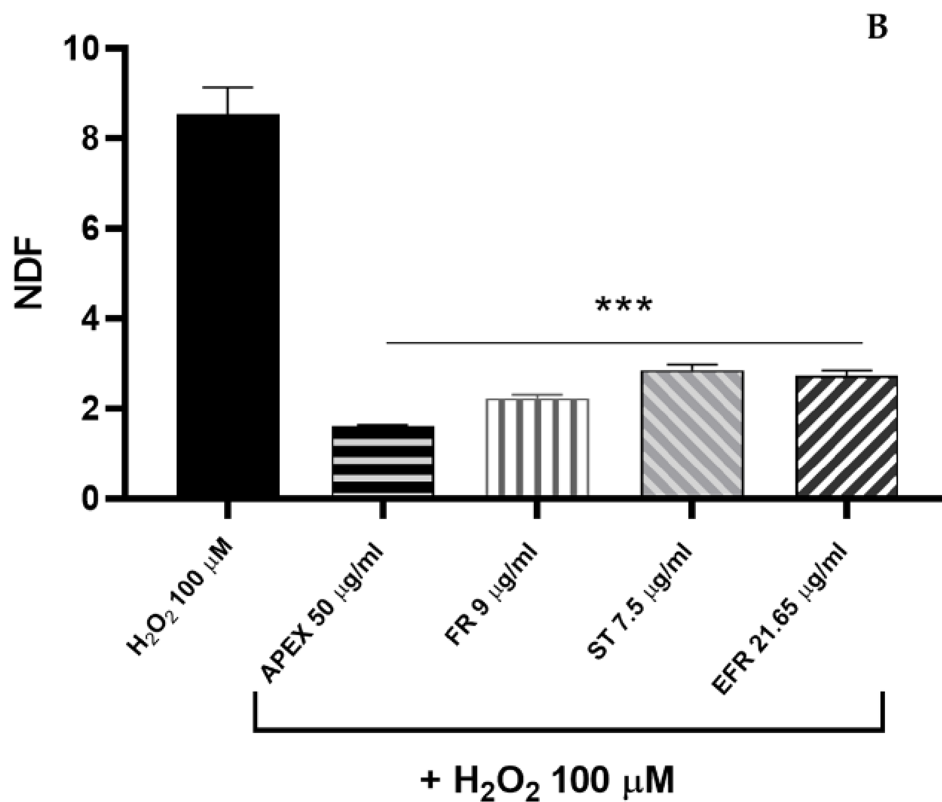


Figure 2.5. Effect of APEX and its major components on oxidant-induced DNA breaks. (A) Representative micrographs of U937 cells exposed to H_2O_2 preincubated for 24 h in the absence or presence of increasing concentrations of APEX; (B) extent of DNA lesions in the nuclear DNA from cells preincubated for 24 with APEX, FR, ST or EFR prior to oxidative challenge. Values are expressed as NDF and are the means ± SEM of three independent experiments. * $p < 0.05$ vs. H_2O_2; *** $p < 0.001$ vs. H_2O_2; *** $p < 0.0001$ vs. H_2O_2.

2.2.4. Anti-Inflammatory Activity

The ability of APEX as well as that of its three main components to counteract the *in vitro* inflammatory effects promoted by lipopolysaccharide (LPS) has been evaluated.

In the first set of experiments we analyzed the gene expression levels of inflammatory markers (namely IL-6, IL-8, TNF-α, IkBα, iNOS) after exposure to LPS (1 µg/ml for 12 hours) in the absence or presence of APEX or of its separate components. As shown in Figure 2.6. and 2.7., a significantly increased expression of all the selected genes could be observed in LPS-treated cells.

Among the concentrations tested, 5 μg/ml APEX exerted a nearly-maximal effect on IL-6 expression (Figure 2.6.) as well as on the other cytokines (not shown): hence 5 μg/ml APEX and the corresponding amounts of FR, EFR and ST (when tested alone) were used in the next sets of experiments. Importantly, pre-treatment with APEX, FR, ST and EFR significantly prevented LPS-induced over-expression of all the selected genes.

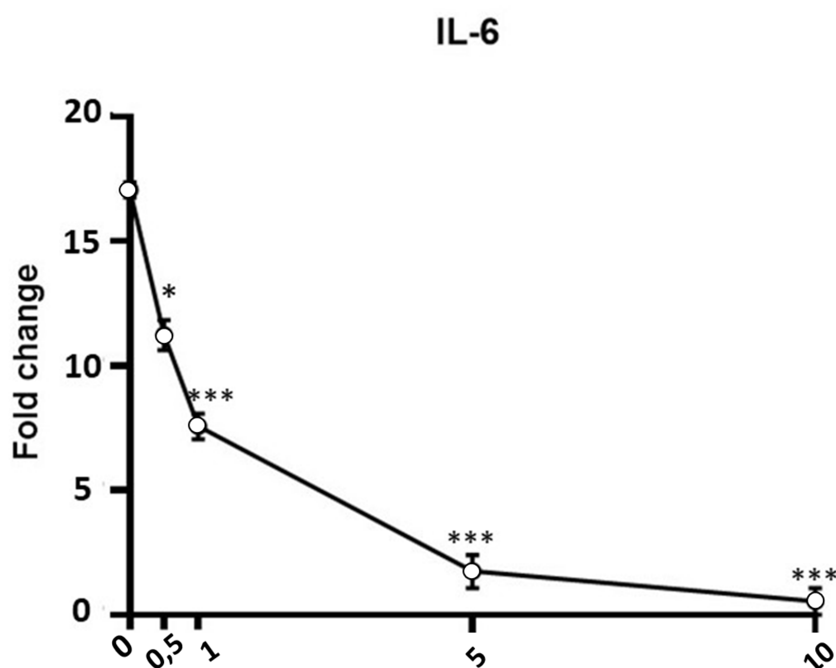


Figure 2.6. Effect of APEX on IL-6 gene expression in LPS-stimulated cells. U937 cells were pre-incubated (24 h) with increasing concentrations of APEX and then grown for further 12 hours with LPS (1 μg/ml); the expression level was determined immediately after LPS treatments. Data are expressed as fold increase as compared to untreated control cells, and are the means ± SEM of at least three independent experiments; *p < 0.05, p**< 0,001,p***< 0,0001, as compared to LPS stimulated cells without APEX (One-way ANOVA).

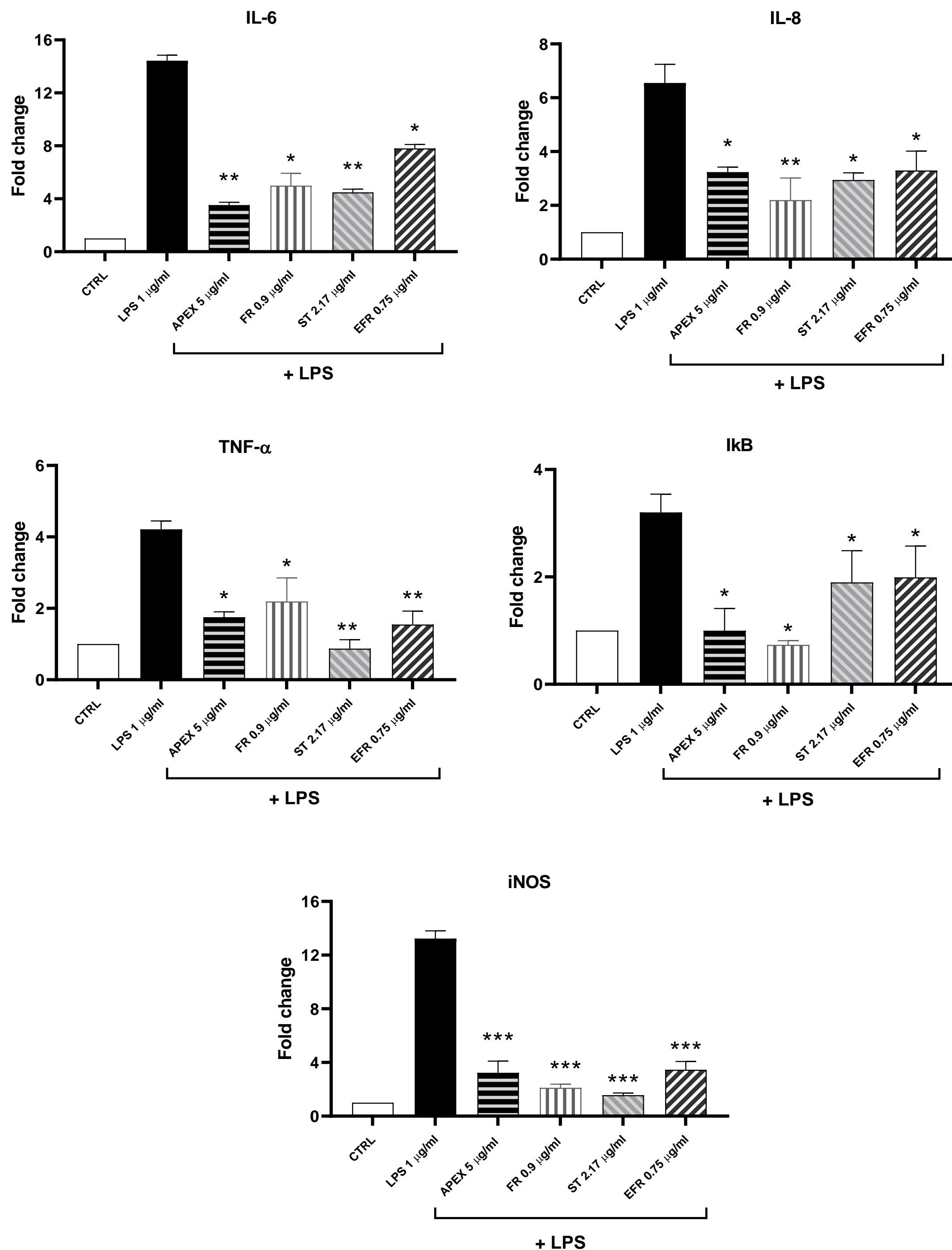


Figure 2.7. mRNA levels of cytokines and selected inflammation markers (IL-6, IL-8, TNF-a, IkBα, iNOS) in LPS-stimulated U937 cells. Cells were pre-incubated (24 h) with APEX, FR, ST and EFR and then grown for further 12 hours with LPS (1 μg/ml); the expression level was determined immediately after treatments.

Data are reported as fold increase as compared to untreated control cells. Data are the means ± SEM of at least three independent experiments; *p < 0.05, p**< 0,001, p***< 0,0001, as compared to LPS stimulated cells.

In order to see whether the LPS-induced increased gene expression resulted in a parallel increase in protein accumulation, the amount of IL-6 released from U937 cells in the growth medium 24 hours after the treatment was determined.
The results of the ELISA assay showed that IL-6 protein levels reflected the gene expression observed in each experimental condition (Figure 2.8.).

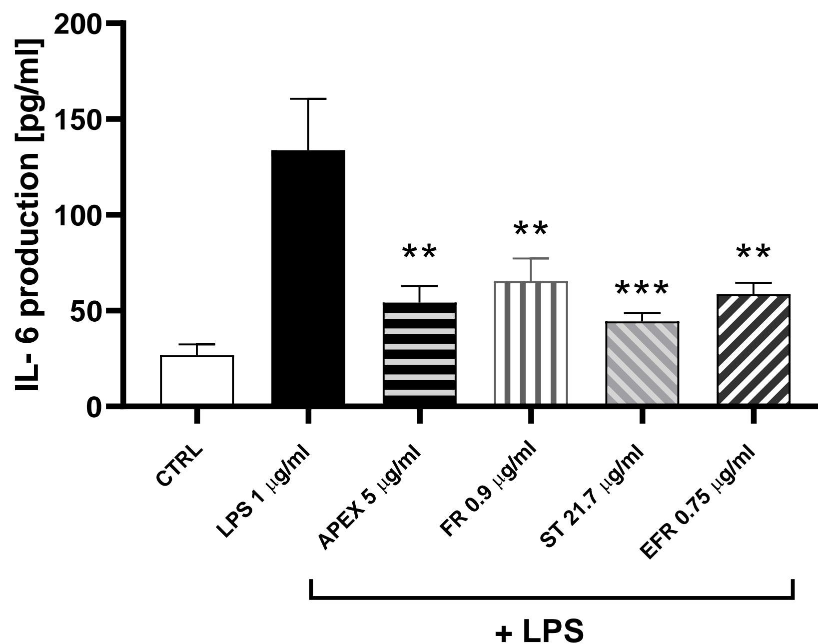


Figure 2.8. Effect of APEX and its major components on IL-6 protein levels. U937 cells were pre-incubated for 24 h with APEX, FR, ST e EFR; at the end of the 24 h 1 µg/ml LPS was added and cells incubated for further 12 h. IL-6 levels were determined after 24 h growth in drug-free medium by ELISA assay. Results are expressed as µg/ml of medium. Values represent the means ± SEM. (n = 3). One-way ANOVA was used to measure the significance of differences between groups. *** $P < 0.001$ LPS vs. APEX and ST treated cells or control vs. LPS vs. FR and EFR treated cells **$P < 0.01$.

To dissect the biological mechanisms at the basis of APEX effects reported above, we next measured the gene expression of the biomarker combination of miR-146a and its target protein IL-1 receptor-associated kinase (IRAK-1) involved in the regulation of IL-6 activation pathway, where IRAK-1 - which actively participates in the Toll-like receptors signaling - is regulated in a negative feedback fashion by miR-146a expression [64]. Therefore, we evaluated the regulation of miR-146a and IRAK-1 in APEX-pre-incubated cells.

We found that IRAK and miR-146a were significantly increased or reduced, respectively, after 12 hours of treatment with LPS (1 μg/ml). Interestingly, APEX promoted an opposite response on LPS-stimulated cells in that it increased the expression of miR-146a while decreasing that of IRAK-1 (Figure 2.9.).

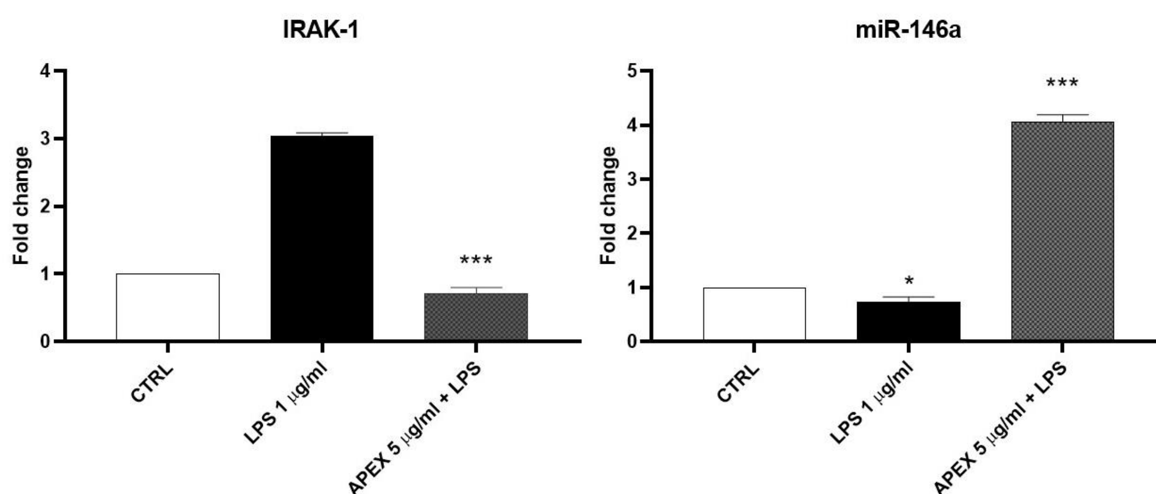


Figure 2.9. Effect of APEX on IRAK-1 and miR-146a expression levels in LPS stimulated U937 cells. Treatments were the same as in Figure 6. Results are reported as fold change as compared to control. Results are the mean ± SEM of three experiments. One-way ANOVA was used: * $p < 0.05$ CTRL vs LPS; *** $p < 0.01$ LPS vs. APEX + LPS

2.2.5. Wound Healing Repair Activity

The correlation between APEX antioxidant/anti-inflammatory effects with its possible ability to heal wounds was performed through the monolayers' scratch assay.

We investigated the wound-healing properties of APEX and its individual constituents on HC-a human chondrocytes (Figure 2.10).

We found that the percentage of wound closure after 24 hours APEX supplementation improved significantly as compared to control cells (91% vs 61%, respectively). Even better results were afforded by FR (99.7%), ST (98.3%) and EFR (96%) treatment (Figure 2.10.).

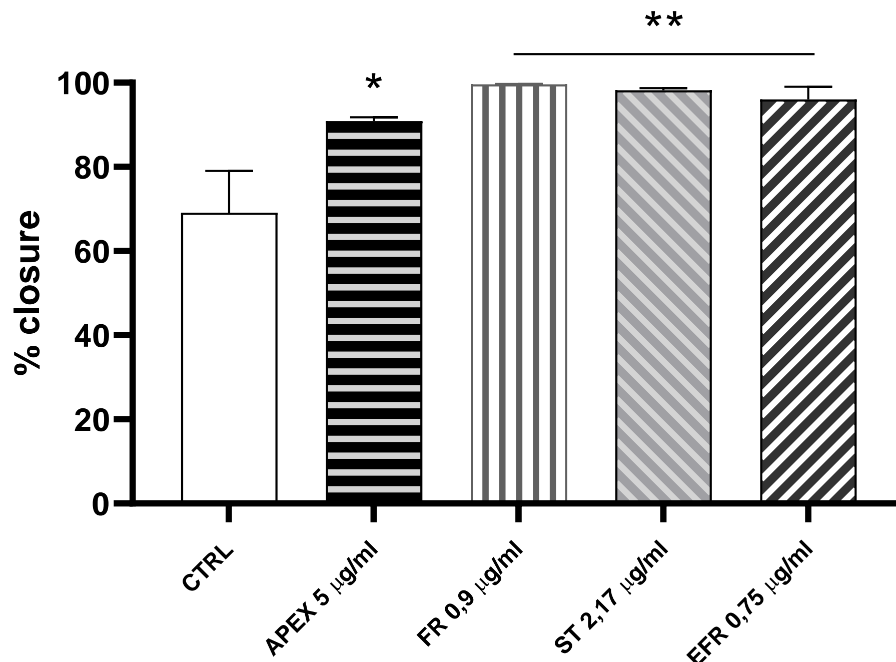


Figure 2.10. Wound-healing after APEX, FR, ST and EFR treatment. The graph shows the percentage of wound closure as compared to the untreated cells HC-a after 24 h. The values represent the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.001$ vs. CTRL (One-way ANOVA).

2.3. Discussion

A variety of products based on the bioactivities of plant extracts or purified compounds are being increasingly developed and globally consumed [65,66] with an estimated value of 140 billion dollars in 2024. Indeed, a wide number of plants and botanicals are capable of affording health benefits mainly consisting in the prevention and/or the adjunctive treatment of some chronic diseases [67]. When extracted from edible plants these compounds are termed as nutraceuticals.

In this context, the present research was undertaken to characterize two relevant biological - namely anti-inflammatory and antioxidant - activities of the ethanolic extract of *C. sativa* roots grown in aeroponics setting.

Aeroponics has been chosen because it allows collecting clean roots free from the parasites and contaminants normally present in soil-cultivated plant [67]; it yields higher amounts of raw material and bioactive constituents; it meets the standards of organic cultivations, which today represent a desired and high-quality end point. Moreover, the aeroponic system is industrially scalable in a cost-effective manner. The composition of APEX (Table 2.2.) had been characterized and compared with the ethanolic extract obtained from soil-cultivated plants in a recent study [67] where it was also found that the per-plant yield of roots' bioactives was higher in aeroponics- as compared to soil-cultivated plants.

In the present work APEX and its components, tested at non-toxic concentrations, have been demonstrated to act as antioxidants in either cell-free or in cell-based determinations.

In acellular systems the antioxidant activity of the extract *in toto* was higher as compared to the single components tested at concentrations equivalent to those present in the whole APEX. This suggests that these molecules do not interact negatively each other, but rather produce an additive-like effect. APEX and its major constituents were active in both DPPH and Fe^{2+} chelation assay indicating

that they act in a bimodal pattern, i.e. through radical-scavenging (direct DPPH quenching) and transition metal-chelation (formation of Fe^{2+} chelates), a mixed mode of action which is rather common in plant-derived bioactive molecules [68].

When tested in cell systems, APEX, FR, EFR and ST retained their antioxidant activity against H_2O_2-induced cytotoxicity and genotoxicity toward U937 cells. This finding lends further support to the above notion that both radical scavenging and iron-chelating capacities contribute to the whole antioxidant activity [68]. However, since it is known that only iron chelators, unlike radical scavengers, are capable of mitigating nuclear DNA oxidative lesions [68], it is plausible that the chelating capacity might have a prominent role in APEX and its constituents antioxidant activity.

When tested separately, the antioxidant activity of FR, EFR and ST in cell systems was comparable to that of APEX, and they did not seem to exert any additive-like effect as observed in cell-free tests. The loss of additive interactions is likely dependent on the fact that - differently from the acellular settings - the relative activity of the compounds given alone or in the phytocomplex may vary significantly, as other components in APEX may for example compete with or affect their cellular pharmacokinetics and pharmacodynamics.

The anti-inflammatory activities of some medicinal plants have been attributed to their contents of terpenes, like FR, that shows a potent in vivo anti-inflammatory activity [47]. Importantly, as recently discussed by Ryz et al. [15] the anti-inflammatory properties of the roots of *C. sativa* have a solid ethnobotanical history and would deserve much more attention. Although there is currently no research available about their relative contribution, FR and EFR are likely candidates accounting for *C. sativa* roots anti-inflammatory activity. Hence, in addition to whole APEX, we addressed our experimental approach on the two triterpenoids and, considering its quantitative prevalence, also on ST.

Here we show that the LPS-stimulated gene expression of relevant inflammation markers, namely IL-6, IL-8, TNF-a, IkBα and iNOS, was efficiently prevented by

APEX; FR, EFR and ST given alone were also effective to a similar extent. Notably APEX promoted these anti-inflammatory responses at concentrations 10 times lower as compared to those protecting toward oxidant insults, suggesting that the first effect is more relevant than the latter.

The anti-inflammatory capacity was further strengthened by the finding that APEX and its major components were all capable to prevent the synthesis and the extracellular-release of IL-6.

Given the importance of IL-6 in inflammation and its recently highlighted implications in COVID-19 pathogenesis [69,70] we investigated the mechanistic interactions of APEX and its selected constituents with the IL-6 gene expression pathway. In particular, we focused on the expression of the miR-146a and IRAK-1. IRAK-1 is involved downstream TLR4 (Toll-like receptor-4) signaling pathway activation; TLR4 represents a key receptor on which different stimuli converge to induce a proinflammatory response. In this condition TLR4 through the IRAK-1 mediator, leads to the activation of the NF-κB transcriptional complex, and finally the production of proinflammatory cytokines, including IL-6. Conversely, miR-146a inhibits the transcriptional complex NF-κB activation and the ensuing pro-inflammatory cytokine production by directly targeting IRAK1 expression [64,71]. According to this notion we found that U937 cells overexpress IRAK-1 while underexpress miR-146a upon LPS stimulation; more interestingly we also found that APEX promotes miR-146a overexpression under the same LPS-stimulus conditions. Hence it is plausible that the miR-146a overexpression may be causally linked to the parallel downregulation of IRAK-1 and mechanistically involved in the overall anti-inflammatory activity of APEX and its major component(s) reported herein.

The fact that other natural products displaying anti-inflammatory capacity have been reported to induce up-regulation of miR-146a [72] lends further support to the above inference.

Hence, APEX shows remarkable antioxidant and anti-inflammatory activities in cultured cells systems. From a quantitative point view, APEX produces its

maximal anti-inflammatory capacity at a concentration 10 fold lower as compared to that resulting in the maximal antioxidant activity. This finding suggests that APEX and its major components might be preferably exploited as anti-inflammatory phytocomplex and natural compounds, respectively. In particular, although this is a mere speculation, the effect of APEX on IL-6 levels via miR-146a/IRAK-1 pathway might be pharmacologically relevant, since it could cooperate with the drugs directly targeting IL-6 such as anti-IL-6 receptor mAbs (e.g., sarilumab, tocilizumab) and anti-IL-6 mAbs [73], which act at a separate, downstream level.

One of the problems affecting the nutraceutical/pharmacological exploitation of phytocomplexes is their scarce absorption and bioavailability through the oral route. Actually, no data are availabe for FR and EFR absorption. However, absorption is not always a limiting factor including, but not restricted to, the topical use for dermatological conditions and the medication of wounds [74].

Indeed chronic inflammatory bowel diseases (CIBD) [75] can be treated with drugs exhibiting scarce or null absorption through the gut as they can act in a topical-like fashion. In this light - nonetheless the lack of information on its pharmacokinetic profile - APEX would be an attractive candidate for the adjunctive management of these severe diseases. Furthermore IL-6 and TNF-α, which are significantly down-regulated and inhibited by APEX, are among the major pathogenetic actors of CIBD [76]; IRAK-1, targeted by APEX, has been found to be involved in ulcerative colitis and Crohn disease, and it is increasingly expressed during the active phases of these conditions [77]. Incidentally, FR has been recently and independently reported to ameliorate chemically-induced colitis in mice through anti-inflammatory mechanisms involving decreased proinflammatory cytokines IL-1β and IL-6 accumulation and inhibition of autophagy via interaction with the AMPK-mTOR signaling pathway [7].

Another clinical setting where pharmacokineetics limitations are circumvented is the infiltrative treatment of arthrosic conditions in which the drugs can be

directly delivered within the articular space [78]. In osteoarthrosis chondrocytes represent a key cellular target of the degradative process. Here we show that HC-a human chondrocytes undergo an accelerated wound closure process in the presence of APEX and its components. Reportedly, the wound closure process leads in the initial phase to an immediate acute inflammation involving different cytokines and growth factors, with IL-6 playing a prominent role [79]. Furthermore, at later stages, a crucial step to achieve complete wound healing is represented by IL-6 down-regulation. Chondrocytes wound closure after monolayers'scratching has been used as a model to mimic chondrocytes' outgrowth necessary in cartilage repair [80]. Notably, when functionally benchmarked in the experimental wound healing assay APEX and its major constituents significantly promoted/accelerated wound closure process, an effect that is likely related according to APEX, FR, EFR and ST anti-inflammatory and antioxidant properties. Along the same line it is important to mention that efficiency of gut mucosa wound closure is known to contribute to the amelioration of the CIBD conditions [81] discussed above.

Another important feature of *C. sativa* roots and of APEX, not secondary to the above considerations, is the very high content of ST [67], whose potential in the adjunctive management of hyperlipidemia and prostate hypertrophy has been fully recognized. In keeping with our data, ST has also been shown to exert remarkable anti-inflammatory effects: indeed, Liao et al. [82] reported that ST suppressed the secretion of inflammatory factors from keratinocytes and macrophages induced by peptidoglycan and significantly reduced the expression of NLRP3, a key component of NLRP3 inflammasomes, and inhibited the activation of caspase-1 and of NF-κB.

Aeroponic roots and APEX may then represent an excellent source of this sterol, far better than the popular *Serenoa repens* extract, which contains a hundred times lower amount of ST [67].

For the sake of completeness, it is important considering that the major limitation of this study is represented by the fact that no in vivo approach has been

included; consequently, the perspectives raised in our study are speculative. However the high anti-inflammatory potency and the fair antioxidant capacity of APEX, along with the rational mechanistic ground proposed herein, are promising with regard to the exploitation of APEX in the above, widespread and socially relevant conditions.

Conclusions

In this research we first assessed the feasibility of aeroponic cultivation of legal varieties of *C. Sativa* L., and its advantages over the traditional in soil cultivation, with particular regard to the exploitation of part of the plants usually considered as waste by-products, such as the roots and, possibly, the amelioration of CBD/Δ^9-THC ratios. Aeroponics allowed us to obtain a robust growth of the plants of both the aerial and radical parts under optimized and strictly controlled conditions. The level of CBD/Δ^9-THC in aeroponics plants inflorescences is still being investigated, while we were able to characterize the chemical composition of the roots, which appeared overgrown and much more easily processable for industrial purposes as compared to plants cultivated traditionally. Roots contained higher amounts, on a per plant basis, of friedelin, epi-friedelanol and β-sitosterol whose co-presence in *C. sativa* roots might envisage their use as preparations for preventive- or adjunctive treatment in several pathological and physiopathological conditions.

The biological activity of APEX *in toto*, our *C. sativa* root ethanolic extract, and of its major constituents was addressed in the second part of this project in which their mild antioxidant- and more prominent anti-inflammatory- effects have been identified.

We have also identified the mechanisms underlying both the actions: the capacity to chelate iron is important with regard to the antioxidant effects of APEX and its major components; as to the anti-inflammation activity the interactions with the expression of miR-146a and IRAK-1, two genes involved in the modulation of IL-6, seems to play an important role.

These properties could be exploited in a wide range of applications in health promotion. The remarkable anti IL-6 activity may even envisage a pharmaceutical application.

Of particular interest might be - once better clarified APEX bioavailability issues - the use of APEX or of powdered aeroponic *C. Sativa* roots in the adjunctive treatment of chronic inflammatory bowel conditions.

On the whole, on the basis of the results obtained, the antioxidant and anti-inflammatory properties of APEX and its bioactive compounds could largely justify their industrial exploitation for the development of pharmaceutical, nutraceutical, cosmeceutical or food products according to desirable sustainability principles.

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Definition of abbreviations

Δ^9-THC: Δ^9-tetrahydrocannabinol

AEP: Aeroponic-elicited plant

AP: Aeroponic plant

APEX: Ethanolic extracts of *C. sativa* aeroponic roots

CBD: cannabidiol

CIBD: chronic inflammatory bowel diseases

DW: Dried weight

EFR: Epi-friedelanol

FR: Friedelin

FW: Fresh weight

RI: Retention index

ST: β-Sitosterol

SP: Soil Plant

List of publications

Included in the thesis

- **Fabio Ferrini**, Daniele Fraternale, Sabrina Donati Zeppa , Giancarlo Verardo , Andrea Gorassini , Vittoria Carrabs , Maria Cristina Albertini , Piero Sestili
  Yield, Characterization, and Possible Exploitation of Cannabis sativa L. Roots Grown under Aeroponics Cultivation
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- **Fabio Ferrini**, Sabrina Donati Zeppa, Daniele Fraternale, Vittoria Carrabs, Giosuè Annibalini, Giancarlo Verardo, Andrea Gorassini, Carmela Fimognari, Maria Cristina Albertini and Piero Sestili
  Characterization of the biological activity of Cannabis sativa L.roots grown under aeroponic system.
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Not included in the thesis

- Sabrina Donati Zeppa, **Fabio Ferrini**, Deborah Agostini, Stefano Amatori, Elena Barbieri, Giovanni Piccoli, Piero Sestili, Vilberto Stocchi. *Nutraceuticals and Physical Activity as Antidepressants: The Central Role of the Gut Microbiota*.
Antioxidants. 2022; 11 (2):236. doi.org/10.3390/antiox11020236

- Giulia Greco, Michael Schnekenburger, Elena Catanzaro, Eleonora Turrini, **Fabio Ferrini**, Piero Sestili, Marc Diederich, Carmela Fimognari. *Discovery of Sulforaphane as an Inducer of Ferroptosis in U-937 Leukemia Cells: Expanding Its Anticancer Potential.*
Cancers. 2021; 14 (1):76. doi.org/10.3390/cancers14010076

- Stefano Amatori, Sabrina Donati Zeppa, Antonio Preti, Marco Gervasi, Erica Gobbi, **Fabio Ferrini**, Marco B. L. Rocchi, Carlo Baldari, Fabrizio Perroni, Giovanni Piccoli, Vilberto Stocchi, Piero Sestili, Davide Sisti. Dietary Habits and Psychological States during COVID-19 Home Isolation in Italian College Students: The Role of Physical Exercise.
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Nutrients. 2019; 12 (1):17. doi.org/10.3390/nu12010017