Optimization of non-psychotropic *Cannabis sativa* L. extraction and evaluation of anti-inflammatory activity on microglial cells

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INTRODUCTION

- ***Cannabis sativa* L. (Cannabaceae)** is an annual flowering plant.

- After the discovery of cannabinoids and of the endocannabinoid system, *C. sativa* is attracting the interest of the scientific community for its potential therapeutic use. Today, **psychotropic cannabis** with high Δ^9-tetrahydrocannabinol (THC) content is enlisted in many official pharmacopoeias. The monograph *Cannabis extractum normatum* was issued in the German Pharmacopoeia in 2020; in Italy leaves and inflorescences and resin are reported in the table II of Farmacopea Ufficiale Italiana XII ed.

INTRODUCTION

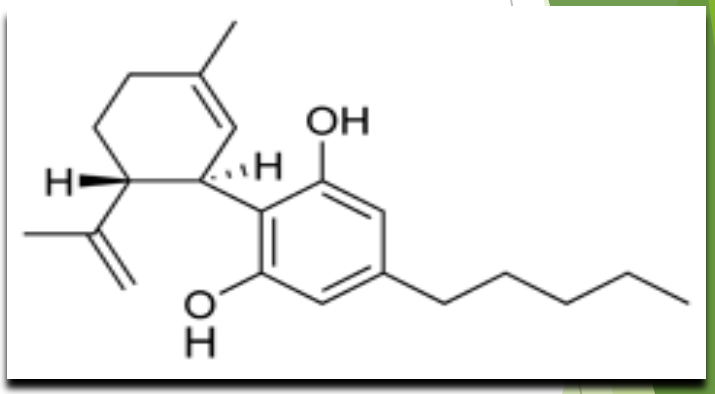
- A vast amount of literature has been published regarding the biological effects of the **non-psychotropic constituents of cannabis, in particular cannabidiol (CBD)**, for the treatment of central and peripheral inflammation, gastrointestinal upset, epilepsy and neurodegenerative diseases.

- Today, the most important limitation in the clinical use of *Cannabis*, particularly for **non-psychotropic cannabis**, is the absence of registered herbal preparations.

NON-PSYCHOTROPIC *CANNABIS*

Cannabinoids

CBD binds the cannabinoid G-protein-coupled receptors (CBR) in the central and peripheral nervous system. Thus, it is indicated for the treatment of some pathologies, such as:



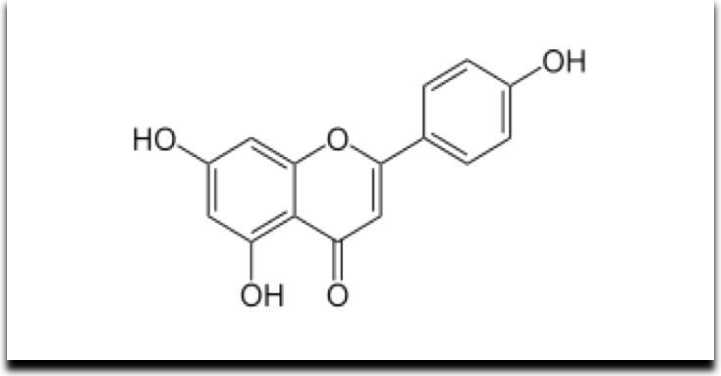
- Periferical and central inflammation
- Psychosis
- Anxiety
- Neurodegenerative diseases as Parkinson, Alzheimer e Huntington disease
- Myocardial and cerebral ischemia
- Cancer, especially to reduce the emetic effects caused by anticancers.

NON-PSYCHOTROPIC *CANNABIS*

Flavonoids

Flavonoids and apigenin have many biological activities, including:

- Anti-inflammatory
- Antioxidant
- Immunomodulation



In particular, the anti-inflammatory activity depends on the apigenin ability to inhibit pro-inflammatory cytokines production, such as TNF-α.

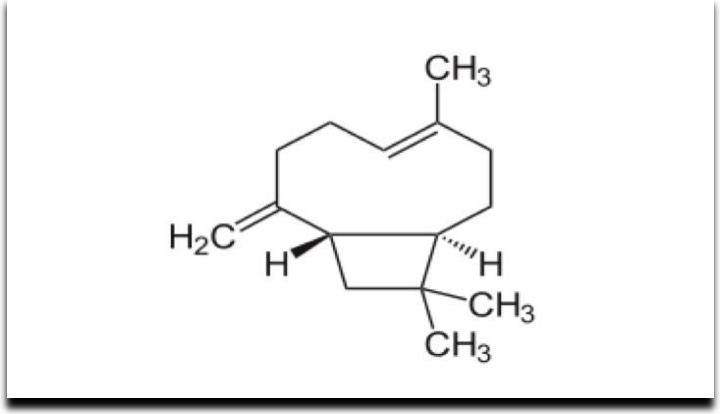
NON-PSYCHOTROPIC *CANNABIS*

Terpenes

The main non-cannabinoid components of cannabis are:

TERPENES:

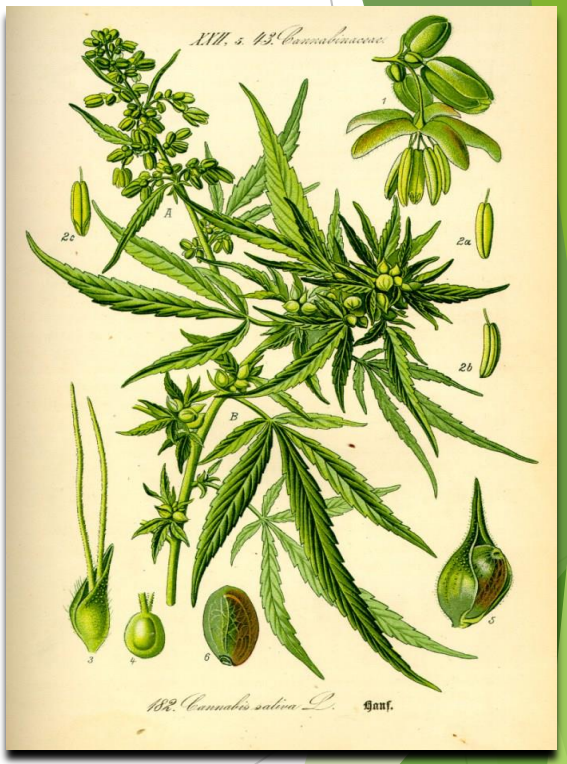
- β-caryiophyllene
- Myrcene
- Limonene



- Anti-inflammatory activity
- Analgesic
- CB2 full agonist (β-caryophyllene)
- Sedative activity

AIM OF THE WORK

- The aim of this work was to **optimize the extraction method of the aerial part (leaves and inflorescences) of non-psychotropic *C. sativa* L. var. *carmagnola*** by using ethanol and olive oil as solvents and by varying the time of extraction and the heat-decarboxylation conditions of the herbal matherial.
- **The main phytocannabinoids and flavonoids in the extracts were analyzed to identify the best extraction method.**



MATERIALS AND METHODS

EXTRACTS PREPARATION

Non-decarboxylated and decarboxylated dried *Cannabis sativa* L. var. *carmagnola* aerial parts (leaves and inflorescences), furnished by CRA, Rovigo, Italy, were extracted using ethanol 96% V/V and extravirgin olive oil (drug:solvent ratio 1:10) .

Extraction methods:

- Maceration with 96% V/V ethanol, according to Farmacopea Italiana FUI XII;
- Percolation with 96% V/V ethanol, according to Farmacopea Italiana FUI XII;
- Extraction with automatic Naviglio Estrattore® (Atlas Engineering, Padova) (96% V/V ethanol);
- Extravirgin olive oil (EVO) maceration according to Romano and Hazekamp method.

MATERIAL AND METHODS

ANALYSIS OF THE EXTRACTS

Qualitative and quantitative analyses of extracts:

- Cannabinoids investigations through HPTLC, using petroleum ether:diethyl ether (80:20) as eluent. The revelation was obtained by means UV lamp at 254 e 366 nm.
- Total polyphenols content (Folin-Ciocalteau assay)
- Total flavonoids content, according to the Ph. Eur. 10 method.

RESULTS

OPTIMIZATION OF THE DECARBOXYLATION TIME

HPTLC analyses revealed a substantial equivalence of acidic to neutral CBD conversion after 60, 120 and 240 minutes of decarboxylation.

Decorboxylation time	HPTLC: CBD spot intensity
30 minutes	+/-
60 minutes	++
120 minutes	++
240 minutes	++

- 60 minutes was considered the best time for cannabis decarboxylation.

EXTRACTS PREPARATION

12 extracts were obtained by means of different extraction methods and different extraction times.

Extraction Solvents:

- ethanol 96% V/V
- EVO (extravirgin olive oil)

Extraction Time:

- 4 hours
- 24 hours
- 72 hours
- 21 days

Extract (DER 1:10)	
Non-decarboxylated Drug	**Decarboxylated Drug**
ethanol 96% V/V: Maceration 4 hours	**ethanol 96% V/V: Maceration 4 hours**
ethanol 96% V/V: Maceration 21 days	**ethanol 96% V/V: Maceration 21 days**
ethanol 96% V/V: Percolation 72 hours	**ethanol 96% V/V: Percolation 72 hours**
ethanol 96% V/V: Naviglio Estrattore® 24 hours	**ethanol 96% V/V: Naviglio Estrattore® 24 hours**
EVO: Maceration 4 hours	**EVO: Maceration 4 hours**
EVO: maceration 21 days	**EVO: Maceration 21 days**

RESULTS

QUALI-QUANTITATIVE ANALYSES OF EXTRACTS

sample	polyphenolic compounds	flavonoids (colorimetric methods)	flavonoids (extraction methods by FUI)	phytocannabinoids
	%	%	%	HPTLC: presence of fluorescent spots at 366 nm
Decarboxylated Drug				
ethanol 96% V/V (DER 1:10) maceration 4 hour	0.054±0.002%	< 0.001%	< 0.001%	+
ethanol 96% V/V (DER 1:10) maceration 21 days	0.078±0.003%	0.003±0.001%	0.003±0.001%	+++
ethanol 96% V/V (DER 1:10) percolation 72 hours	0.083±0.003	0.003±0.001%	0.001±0.001%	++
ethanol 96% V/V (DER 1:10) Naviglio Estrattore 24 hours	0.061±0.004%	< 0.001%	0.001±0.001%	++
EVO (DER 1:10) 4 hours	0.088±0.002%	< 0.001%	< 0.001±0.002%	+/-
EVO (DER 1:10) 21 days	0.106±0.004%	< 0.001%	< 0.001±0.002%	+/-
Non-decarboxylated Drug				
ethanol 96% V/V (DER 1:10) maceration 4 hours	0.075±0.002%	< 0.001%	< 0.001±0.002%	+
ethanol 96% V/V (DER 1:10) maceration 21 days	0.073±0.003%	0.007±0.001%	0.003±0.001%	+
ethanol 96% V/V (DER 1:10) percolation 72 hours	0.079±0.003%	0.007±0.001%	0.003±0.001%	++
ethanol 96% V/V (DER 1:10) Naviglio Estrattore 24 hours	0.057±0.002%	0.003±0.001%	0.001±0.001%	+
EVO (DER 1:10) 4 hors	0.105±0.004%	< 0.001%	< 0.001%	+/-
EVO (DER 1:10) 21 days	0.105±0.004%	< 0.001%	< 0.001%	+/-

RESULTS

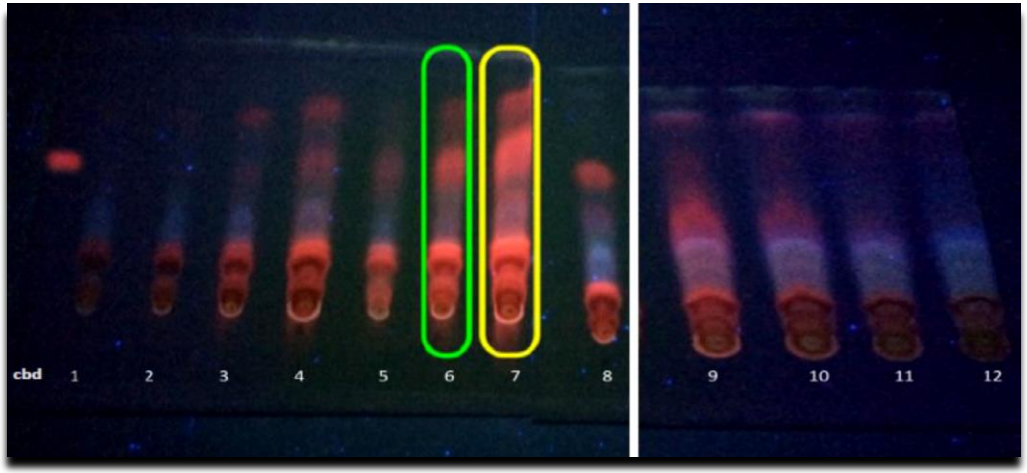
HPTLC

Semiquantitative analysis of cannabinoids.

Rf comparison of the extracts with the standard CBD (**Rf = 0.65**).

The first 4 samples starting on the left are non-decarboxylated samples and the next 4 the corresponding decarboxylates.

The most intense stain at Rf = 0.65 was found in samples 6 and 7, corresponding to Naviglio Estrattore® and maceration after 21 days, respectively, from decarboxylated hrrbal drug.



HPTLC analysis highlighted that ethanolic extracts (samples 1-8) are richer in cannabinoid than the EVO preparations (samples 9-12).

RESULTS

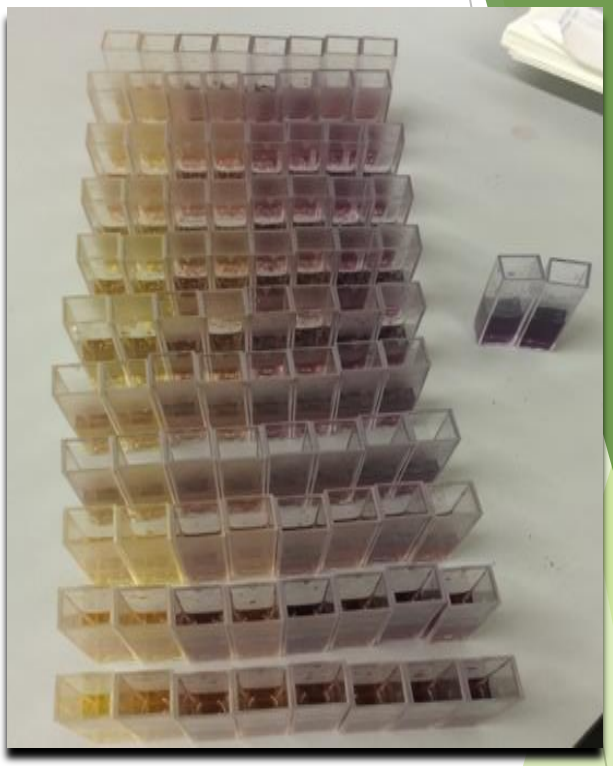
OPTIMIZATION OF EXTRACTION METHOD

- Herbal drug should be decarboxylated in oven at 145 °C for 60 minutes.
- The 96% V/V ethanolic extract (DER 1:10) obtained by maceration for 21 days of the decarboxylated herbal material gave the best yield of CBD and flavonoids.
- Automatic extraction method in ethanol 96% V/V for 24 hours by means of Naviglio Estrattore® gave a good yield of active compound.

RESULTS

DPPH TEST

sample	DPPH inhibition (IC_{50}) %
Decarboxylated Drug	
ethanol 96% V/V maceration 4 hour	2.41±0.21%
ethanol 96% V/V maceration 21 days	1.87±0.14%
ethanol 96% V/V percolation 72 hours	1.98±0.20
ethanol 96% V/V Naviglio Estrattore® hours 24 hours	2.16±0.09%
Non-decarboxylated Drug	
ethanol 96% V/V maceration 4 hours	1.57±0.11%
ethanol 96% V/V maceration 21 days	2.04±0.19%
ethanol 96% V/V percolation 72 hours	2.16±0.15%
ethanol 96% V/V Naviglio Estrattore® 24 hours	2.55±0.18%

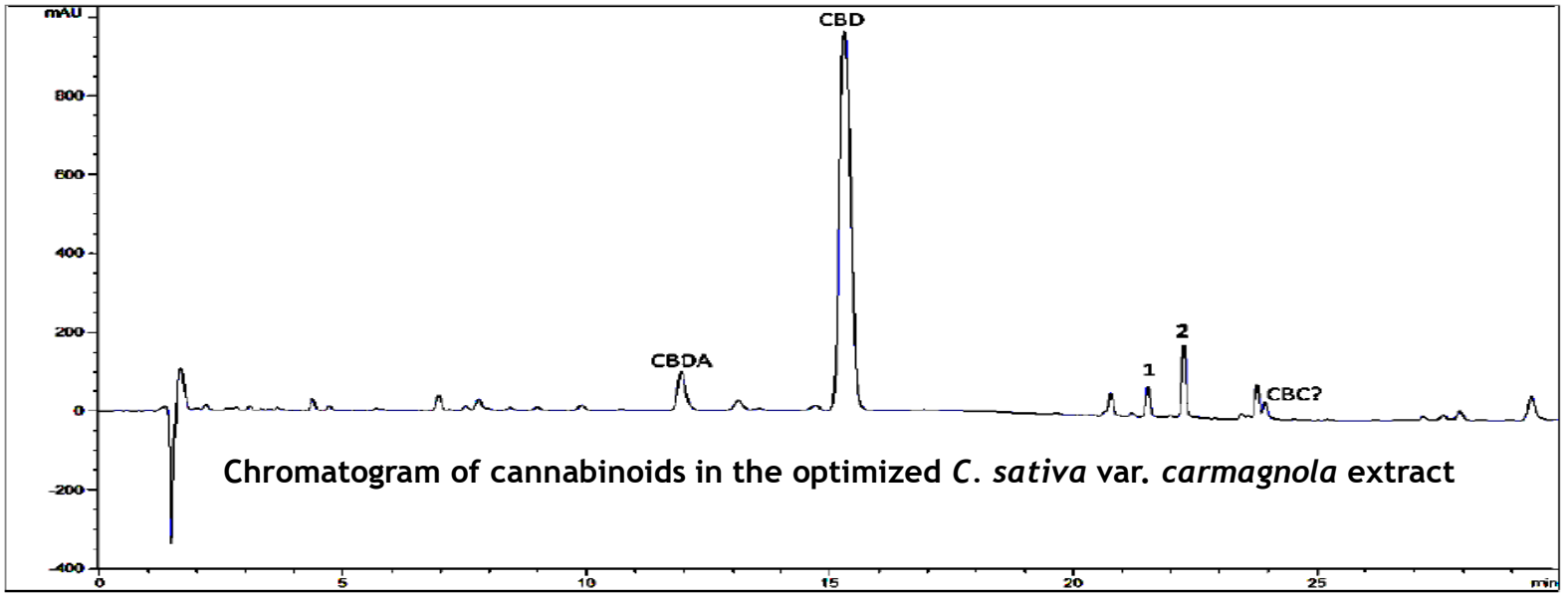


- A good antiradicalic capacity was observed for all tested samples, with IC_{50} ranging from 1.57% to 2.55%.
- There were no significant differences between samples obtained from decarboxylated drug and those obtained from non-decarboxylated drug.

RESULTS

HPLC-DAD
CBD E VITEXIN QUANTIFICATION

sample	Total polyphenols	total flavonoids	CBD	vitexin	monoterpenes
			mg/l		
ethanol 96% V/V maceration 21 days	**783.7±33.5**	**85.0±10.0**	**466.10±2.7**	**12.2±0.5**	**< 0.1**



Chromatogram of cannabinoids in the optimized *C. sativa* var. *carmagnola* extract

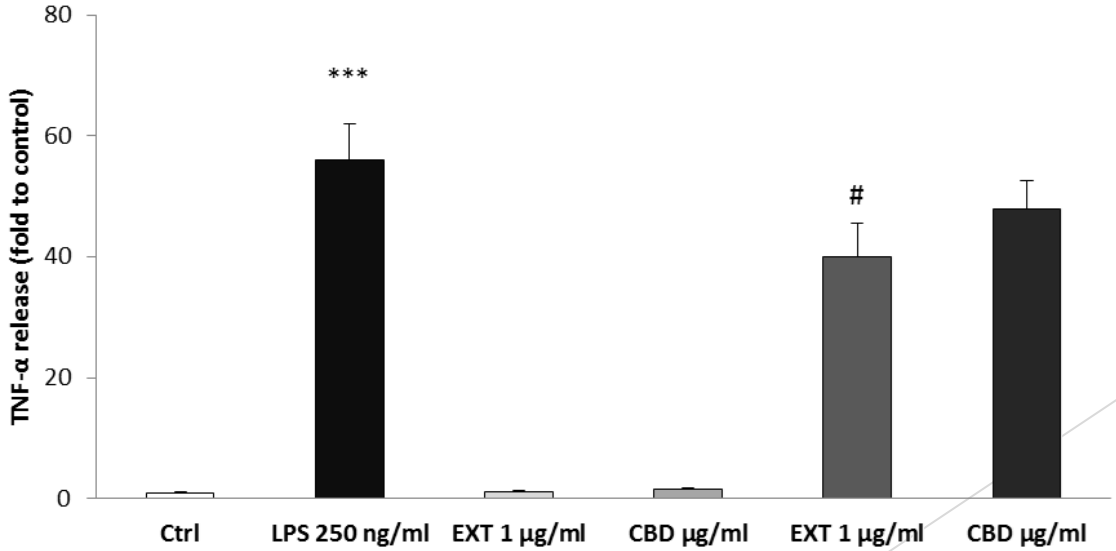
ANTI-INFLAMMATORY ACTIVITY IN AN *IN VITRO* MODEL OF NEUROINFLAMMATION

The optimized extract (EXT) was tested in an *in vitro* model of neuroinflammation, using BV2 microglial cells.

Cells were pretreated with EXT (1 µg/ml) or CBD (1 µg/ml) and then exposed to the inflammatory stimulus (LPS 250 ng/ml) for 2 hours.

The production of TNF-α was quantified by means of non competitive sandwich ELISA.

EXT, but not CBD alone, significantly reduced the LPS-induced production of TNF-α compared to LPS alone.



CONCLUSIONS

- *C. sativa* should be not considered just a fashion or a psychoactive drug, but it can be actually considered one of the most promising medicinal plant for painkillers non-responsive patients and for neuro-inflammatory diseases. Nevertheless, the actual potential of cannabis phytocomplex has yet to be studied, being not only represented by cannabinoids. It is crucial to define and optimize the extraction method in order to preserve the phytocomplex and this study represents our first effort on the topic.

- The maceration for 21 days, using ethanol as a solvent, as described by the Italian Pharmacopoeia XII ed., was the best condition for extracting the non-decarboxylated *Cannabis sativa* L. var. *carmagnola* aerial parts, resulting in a polyphenols and cannabinoid rich extract which has an interesting anti-inflammatory activity on microglial cells.