Pharmacological activity of an *Eupatorium cannabinum* L. extract

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**Abstract**
Pharmacological activity of *Eupatorium cannabinum* L. hydroalcoholic extract have been studied with the use of two in vitro assays - DPPH reduction spectrophotometric assay, and evaluation of cytotoxic effect on Jurkat cells by MTS assay. Also, HPTLC fingerprint and HPLC analysis were performed in order to elucidate the chemical composition of the extract. Thus, it was found that the extract contains 56.86mg% caffeic acid, 1.26mg% eupatorin, 18.06mg% eupatilin, 97.03mg% quercetin, 762.63mg% rutin, 68.83mg% \( \beta \)-ecdysone, among other unidentified compounds. It was showed the dose-response dependency in the DPPH assay, the extract exhibiting an inhibition of DPPH radical of 59% at 3mg/mL concentration, comparing to caffeic acid (0.1mg/mL) used as standard. In the cytotoxicity assay on Jurkat cells, the extract (7-500 µg/mL) showed a dose-response cytotoxic effect, both at 24 and 48 hours.
Moreover the single-dose toxicity test proved that the extract is non-toxic at the dose of 125mg/20gw and it is safe to use.

This study contributes to the knowledge of *E. cannabinum*, add a scientific proof of the known traditional uses and, in the same time, opens the way for further investigations.

**Keywords: Eupatorium cannabinum** L., scavenger, cytotoxic

**Introduction**
The genus *Eupatorium* belongs to the Eupatorieae, one of the 13 tribes of the Asteraceae, and comprises of nearly 1200 species distributed mainly in the tropical regions of Americas, Europe, and Asia (ZHANG & al. 2008 [1]). *Eupatorium cannabinum* L. (hemp agrimony) is a herbaceous plant common in Europe, Central Asia and North Africa, preferring damp places near water. It shows a strain of up to 1.5 m high, leaves with 3-5 sections having unevenly toothed leaflets, pubescent and white or pink flowers, gathered in corymbiform calathidiums (ISTUDOR [2]).

Although the reported chemical constituents and pharmacological potential of *E. cannabinum* are scarce, it is well known that the chemical composition of the genus *Eupatorium* include monoterpenic derivatives, sesquiterpenes, diterpenes, triterpenes, flavonoids, pyrrolizidine alkaloids, essential oil, and some others, all of these compounds exhibiting various biological activities.

Analytical studies of *Eupatorium cannabinum* volatile oil led to the identification of 64 components that represent more than 94% of the total, among them are: (Z)-2-hexene 2 pentylfuran, benzaldehyde, \( \beta \)-pinene, \( \delta \)-2-carene, \( \alpha \)-phellandrene, \( p \)- and \( o \)-cymene, limonene, 1,8-cineole, phenylacetaldehyde, (E)-\( \beta \)-ocimene, \( \gamma \)-terpinene, terpinolene, 2-phenylethanol, linalool, \( \alpha \)-terpineol, methyl thymol, carvacrol methyl, indole, thymol, \( \alpha \)-cubeben, neryl acetate, \( \beta \)-bourbonen, \( \beta \)-cubebene, \( \beta \)-caryophyllene, \( \alpha \)-santalene, \( \beta \)-gurjunene, \( \alpha \)-humulene, germacrene D, valencene, bicyclogermacrene, \( \alpha \)-farnesene, \( \gamma \)-cadinene, \( \delta \)-cadinene, \( \beta \)-
sesquifelandren, germacren B elemol, ledole, caryophyllene oxide cedrol, hexadecane, \( \gamma \)-eudesmol, T-cadinol, \( \alpha \)-cadinol, \( \beta \)-bisabolol, heptadecane, octadecane, phytol, palmitic acid. Out of these, 32 compounds are hydrocarbons (77.1%) and 29 are oxygenated compounds (16.8%); sesquiterpenes are prevalent (72.4%) while monoterpenes represent only 17.4% of all total compounds. The most important constituents of the sesquiterpenes fraction are germacrene D (33.5%), \( \alpha \)-farnesene (12.9) and the oxygenated compounds elemol (2.8%) and \( \alpha \)-cadinol (2.7%) are the most common. Oxygenated monoterpenes represent only 1.6% of oil content and the most important representatives are linalool and \( \alpha \)-terpineol. Of hydrocarbon monoterpenes (16.5%), the most important compound is \( \delta \)-2-carene (6.5%) (SENATORE & al. [3]).

The volatile oil also contains sesquiterpenoid lactones of germacranolides group: eupatolide and 0.4% eupatoriopycrin.

Among flavonoidic compounds present in *Eupatorium cannabinum* are known: heterosides of eupatorin, hyspidulin, kaempferol and quercetin; astragalin, kaempferol-3-rutinoside, hyperoside; izoquercitrin (ELEMA & al. [4]).

Fraissee et al, reported the composition in polyphenols of *E. cannabinum*: chlorogenic acid 14.67±0.73 (g/kg on dry matter); 3,5-dicaffeoylquinic acid 22.74%±1.13 (g/kg on dry matter); 4,5-dicaffeoylquinic acid 4.23±0.23 (g/kg on dry matter); 41.64 (g/kg on dry matter) total caffeoyl derivatives (determined by HPLC), 65.72±3.3 (g/kg on dry matter) total dihydroxycinnamic derivatives (determined by colorimetry), 8.10±0.41 (g/kg on dry matter) total flavonoids (colorimetry); 73.82 (g/kg on dry matter) total dihydroxycinnamic derivatives + total flavonoids (calculated by summing); 81.47±3.75 (g/kg on dry matter) total polyphenolic compounds (colorimetry) (FRAISSE & al. [5]).

In Europe, *Eupatorium cannabinum* L., is a species commonly used in traditional medicine as a good choleretic, laxative, diuretic and hypocholesterolemic (LEXA & al. [6]) It is also used to treat skin diseases such as psoriasis, eczema, boils. (FLAMINI & al [7])

Pharmacological activities such as immunological, antibacterial and cytostatic are determined by the presence of flavonoids (STEVENS & al. [8]), polysaccharides (VOLLMAR & al. [9]), nontoxic alkaloids, sesquiterpenoid lactones and volatile oil. (WOERDENBAG & al. [10]).

Antimicrobial activity of the essential oil was evaluated in vitro on 8 Gram + and Gram - species: *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Salmonella typhi* Ty2. The results show a significant antimicrobial activity against all microorganisms studied but especially on Gram + species and in particular *Streptococcus faecalis*. *Pseudomonas aeruginosa* showed the highest resistance to volatile oil. (SENATORE & al. [3]).

Recently, the plant has been found of use as an immune system stimulant, helping to maintain resistance to acute viral and other infections. A homeopathic remedy is made from the leaves. (FU & al. [11])

Ahead of flavonoids, the main caffeoyl derivatives among polyphenols are considered the major antioxidant compounds in the aerial parts of Asteraceae herbs (FRAISSE & al. [5]). 20-hydroxiecdysone, an ecdysteroid hormone, exhibits antioxidative effects (HU & al. [12]), protects against cerebral ischemia injury by inhibiting ROS/RNS production and modulating oxidative stress-induced signal transduction pathways.

The aim of this study is to evaluate the scavenging and cytotoxic potential of *E. cannabinum*. 
Materials and methods

Plant material

*Eupatorium cannabinum* L. was cultivated in ecological conditions by a commercial grower (Dacia Plant, Bod, Romania) in 2012, dried and ground as a fine powder. A voucher specimen was deposited at the manufacturer.

Chemicals and apparatus

2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH derivative), quercetin, hyperoside, rutin, chlorogenic acid and caffeic acid, were purchased from Sigma Aldrich-Fluka (St. Louis, MO). Eupatorin, eupatilin and β-ecdysone were purchased from PhytoLab (Baden-Wurttemberg, Germany). For absorbance measurements, a Helios Gamma UV/VIS spectrophotometer was used. A HPTLC scanner with computer system and WinCats Version Software were obtained from Camag (Muttenz, Switzerland). Camag Linomat V was used as applicator. Separation was done on silica gel F254 HPTLC precoated plates purchased from Merck (Germany).

For *in vitro* assay, Jurkat cell line was purchased from ATCC, CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was provided by Promega, USA, all other materials for maintaining the cell culture were from Sigma Aldrich-Fluka and the readings were performed on a LKB Chameleon microplate reader.

Samples preparation

20 g of raw material in 20 mL of 70% ethyl alcohol were soaked for 10 days at room temperature in a dark place and then filtrated. The crude extract was concentrated under reduced pressure (72-74 mmHg) and the resulted spiss (3,5 g) was dissolved in 20% propyleneglycol to obtain a stock solution of 1g/ mL. The extract was further diluted in the same solvent according to each assay protocol.

HPTLC analysis

Chromatography was performed on silica gel F254 HPTLC pre-coated plates. Samples were applied on the plates as band of 7mm width using a Camag Linomat V sample applicator at the distance of 14mm from the edge of the plates. The mobile phase was constituted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v). After development, plates were dried and derivatised in NP-PEG reagent. The fingerprints were evaluated at 366nm in fluorescence mode with a WinCats and VideoScan software. Reference compounds for HPTLC analysis were caffeic acid, chlorogenic acid, rutin, hyperoside (10⁻³ M).

HPLC analysis

Chromatographic separation was achieved on a HPLC ELITE – LaChrom system, with DAD detector; a Luna C18(2) column (250 x 4.6 mm, 5µm) at 23°C, using a gradient elution. Separation of polyphenols was performed using a mobile phase consisting of an A solution (water acidified with phosphoric acid, pH = 3.0) and a B solution (acetonitrile) at an initial flow rate of 1 mL/min; with an injection of 10 µL.

For determination of ecdysones content the above mentioned column was used and a mobile phase consisting of 20% acetonitrile for isocratic elution at an initial flow rate of 1,5 mL/min; with an injection of 20 µL .

Free radical scavenging assay

The extract was diluted in methanol at the concentration of 10, 7, 5, 3, 2, 1 and
0.1mg/mL. 50 μl aliquots of the extract were mixed with 2950 μl of the methanolic DPPH-derivative solution (0.025g/L). The reduction of the DPPH free radical was measured by reading the absorbance at 517nm and related to the absorbance of the control without the herbal drugs. Inhibition ratio (percent) was calculated from the following equation: 

\[
\% \text{ inhibition} = \left( \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100\%.
\]

Caffeic acid methanolic solution (0.1mg/mL) was used as positive control.

**Cytotoxicity assay**

The cytotoxic effect of the *Eupatorium* extract on Jurkat T cells (a leukaemic T-cell line) was analyzed by a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, reflecting cell viability, as described in the manufacture kit. For the MTS assay, Jurkat T cells (2.5 × 10^5 per well) were incubated with extract of several dilutions, phytohemagglutinin (PHA, 0.1%) or 5-fluorouracil (5-FU, 200μg/mL) in 96-well plates. After incubation for 24 h and 48h, 50 μl of the MTS solution was added to each well and incubated for an additional 4 h. The optical density (OD) values of the solutions were measured at 492 nm using a plate reader.

All data are expressed as the mean ± SD. Statistical analysis was done using student’s t-test. Dose-response curves between cell viability percentages and extract concentrations were constructed. The cell viability (%) was calculated as follows:

\[
\text{Cell viability} (\%) = \left( \frac{\text{Mean OD of treated cells}}{\text{Mean OD of control cells}} \right) \times 100
\]

**Single dose toxicity**

Single dose toxicity test is a qualitative and quantitative study revealing toxic phenomena and characterizing their appearance over time after administration of a substance or combination of substances in a single dose. In accordance with regulations on single dose toxicity test, it is not necessary to have a high level of precision in the quantitative assessment (eg LD50), but a maximum tolerated dose (MTD) and it is recommended to get a maximum of relevant information with reduced number of animals (SENATORE & al. [3])

NMRI male mice (18-20g) were purchased from Cantacuzino Institute animal house. The animals were kept under controlled condition (temperature: 22±2°C, humidity: 50–60%, 12h light-dark cycle) and had free access to food and water. The animals were acclimatised for 7 days to the laboratory conditions before doing experiments and were deprived of food 12 hours before doing experiments.

The experiment was carried out in accordance with the guidelines for the care of laboratory animals and ethical guidelines, and were approved by the ethics committee for research on laboratory-animal use of the institution. The number of animals was the minimum necessary to show consistent results.

The extract was given orally, by intragastric gavage, 0.5 ml/20 gw (25 ml/kgw).

After administration, the animals were monitored and observed every hour the first day, and by the end of 14 days were subjected to observation at least twice a day. They have been weighed weekly.

**Results and discussions**

**HPTLC analysis**

The fingerprint of the constituents present in sample was recorded using Camag TLC
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visualizer and WinCats Software. The chromatogram (Figure 1) showed characteristic spots for polyphenolic compounds. In particular, chlorogenic and caffeic acid appear as intense fluorescent blue spots (Rf-value 0.47 and 0.96, respectively). Quercetin glycosides are also present in the *Eupatorium* extract as follows: rutin at Rf~0.38 and hyperoside at Rf~0.62.

![HPTLC fingerprint of E. cannabinum extract, after derivatization with NP-PEG, 366nm (a). HPTLC profiles for reference substances and vegetal extract (b)](image)

**HPLC analysis**

High performance liquid chromatography (HPLC) was performed for the analysis of polyphenolic compounds and other major active compounds in *E. cannabinum* hydro-alcoholic extract (Figure 2). The content of chemical constituents was expressed on the dry extract weight basis. Thus, it was found that the extract contains 56.86mg% caffeic acid, 1.26mg% eupatorin, 18.06mg% eupatilin, 97.03mg% quercetin and 762.63mg% rutin. By another method of investigation, it was found that the extract contains 68.83mg% β-ecdysone, among other unidentified compounds.

![HPLC chromatogram of E. cannabinum hydro-alcoholic extract](image)

**Figure 2.** HPLC chromatogram of *E. cannabinum* hydro-alcoholic extract
Stationary phase—Luna C18(2) column (250 x 4.6 mm, 5µm); mobile phase-water acidified with phosphoric acid, acetonitrile; flow-rate of mobile phase-1 ml/min; detection wavelength-254 nm; injection sample volume-10 µl

**Free radical scavenging assay**

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to discolor in the presence of antioxidants. Comparison of the scavenging activity of the extract (at doses of 0.1-10mg/mL) and control (20% propylene glycol instead of extract) is shown in Figure 3. The reference standard (caffeic acid) showed inhibition activity of 57±1.34% at 1mg/mL concentration. EC50 of the extract was determined at 2.91mg/mL.

![Scavenging activity of E. cannabinum extract](image)

Fig. 3. DPPH scavenging activity of *E. cannabinum* extract at different doses. Results are mean ± SD (n=3)

**Cytotoxicity assay**

We examined the viability of Jurkat cells treated with increasing concentrations of *E. cannabinum* hydro-alcoholic extract (7- 500 µg/mL) for 24 and 48 hours using the MTS assay, as described in Materials and Methods. The extract significantly inhibited the growth of Jurkat cells in a dose- and time-dependent manner, especially when treated for 48 hours, as illustrated in Figure 4. Cell proliferation consistently decreased, from 60.64±0.06% viability for the 125µg/mL dose at 24 h to 26.51±0.013% for the same concentration at 48 h. The inhibitory capacity of *E. cannabinum* extract at the dose of 250 µg/mL was comparable to that of 5-FU (200µg/ mL), a well-known anti-cancer agent. The IC50 value of the extract was determined at 48 hours (73.3 µg/mL). The cell culture viability was not affected by the administration of 0.1% PHA, a myogenic lectin.
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**Single dose toxicity**

Administration of 125mg/ 20gw of the extract conducted to a reduced spontaneous motility, drowsiness, ptosis. Phenomena were spontaneously reversible and no other behavioral changes were noticed by the end of the observation period. No deaths were recorded.

**Conclusions**

*Eupatorium cannabinum* is an indigenous species with high pharmacological potential, not very well studied until present. In this study, we analyzed a hydro-alcoholic extract, first from the chemical point of view, and then we investigated some potential pharmacological applications. By both qualitative and quantitative methods, it was showed that the extract contains a high amount of rutin (762.63mg%), other known polyphenolic compounds such as caffeic acid and also methoxylated flavones – eupatorin and eupatilin. The content of $\beta$-ecdysone was also investigated, due to its antioxidant potential, and it was found that the extract contains an important amount (68.83mg%).

As regards the pharmacological potential of the extract, two assays were conducted, one for determination of the scavenging potential (DPPH method) and the other for evaluation of cytotoxic activity on Jurkat cells. It was showed the dose-response dependency in the DPPH assay, the extract exhibiting an inhibition of a DPPH-derivative radical of 59±2.45% at 3mg/ mL concentration, comparing to caffeic acid (0.1mg/ mL) used as standard. In the cytotoxicity assay on Jurkat cells, the extract (7- 500 µg/mL) showed a dose-response cytotoxic effect, both at 24 and 48 hours.

It can be assumed that the varied chemical composition of the extract, rich in polyphenol and sterolic compounds (responsible of the antioxidant effect), bitter principles such as eupatolide and eupatoriopicrin (which are, probably, responsible of the cytotoxic effect as suggested Woerdenbag in 1986) contribute to the beneficial effects of this extract. Moreover the single-dose toxicity test proved that the extract is non-toxic at the dose of 125mg/ 20gw and it is safe to use.
This study contribute to the knowledge of *E. cannabinum* species, add a scientific proof of the known traditional uses and opens in the same time the way for further investigations.

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