Evaluation of antiproliferative and protective effects of *Eupatorium cannabinum* L. extracts

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**Abstract:** *Eupatorium cannabinum* L. (Asteraceae) has been used for a long time for medicinal purposes due to its various pharmacological effects and richness in active compounds such as phenolics, sesquiterpenes, pyrrolizidine alkaloids, and polysaccharides. Despite the high content of compounds that have important roles in medicinal plants, there are still limited literature data regarding this valuable species. The plant was fractioned using chloroform (EC) and distilled water (EA) and HPLC analysis revealed the presence of eupatorin, eupatilin, and quercetin in EC and caffeic acid and rutin in EA. The antiproliferative potential on BT-20, HepG2, Caco-2, and Jurkat cancer cell lines was assessed by MTS test. Jurkat cells were more sensitive to both extracts (IC₅₀ of 7.35 ± 0.35 for EC and 13.77 ± 2.16 µg/mL for EA), while the other lines were susceptible only to EC (IC₅₀ 88.27 ± 1.34 on Caco-2 cells and over 100 µg/mL on BT-20 and HepG2 cells) after 24 h of exposure. In an LPS-induced damage mouse model of endotoxemia, we showed that preventive administration increases the survival times of mice and leads to inhibition of proinflammatory cytokines. Both polar and nonpolar compounds are involved in exerting these effects, but further analytical studies are needed to identify the key responsible compounds and their biochemical pathways.

**Key words:** Hemp-agrimony, antiinflammatory, methoxyflavones, endotoxemia

1. Introduction

The genus *Eupatorium* belongs to the family Asteraceae and comprises about 60 species that have mostly been used in folk medicine or as ornamental plants. Among species, many possess a wide range of pharmacological activities, such as cytotoxic, antifungal, insecticidal, antibacterial, antiinflammatory, and antinociceptive activities (Li et al., 2015). For some species like *E. perfoliatum*, *E. chinense*, *E. arnottianum*, and *E. lindleyanum*, these diverse therapeutic indications are correlated with specific active compounds such as phenolics, sesquiterpenes, pyrrolizidine alkaloids, and polysaccharides.

Although *Eupatorium cannabinum* L., commonly known as hemp-agrimony, has been used for a long time for medicinal purposes, few scientific studies have occurred. In this regard, some studies have been conducted for chemical analysis, focusing especially on essential oil constituents (Senatore et al., 2001; Paolini et al., 2005; Mirza et al., 2006), and some identified compounds of other classes such as polysaccharides (Vollmar et al., 1986), pyrrolizidine alkaloids (Hendriks et al., 1983, 1987), polyphenolcarboxylic acids (Fraisse et al., 2011; Ionita et al., 2013), and flavones (Elema et al., 1989).

Considering the biological activity of *E. cannabinum*, however, few studies are available related to antitumor (Chen et al., 2014; Ribeiro-Varandas et al., 2014), antiinflammatory (Chen et al., 2011), hepatoprotective (Lexa et al., 1989; Clavin et al., 2007; Judzentiene et al., 2016), and immunomodulatory (Vollmar et al., 1986; Clavin et al., 2007) properties of crude extracts or isolated compounds.

The ethanolic extract was more studied, and reports showed both cytotoxic and antiinflammatory activity. Ribeiro-Varandas et al. (2014) showed that *E. cannabinum* had cytotoxic activity on HT29 cancer cells and synergistic effects with doxorubicin. Similarly, the extract modulates
proinflammatory functions of stimulated neutrophils such as reactive oxygen species, interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α) production/release, and expression of adhesion molecules CD11b/CD18. The major sesquiterpene lactone isolated from this species, eupatoriopicrin, inhibits (IC_{50} < 1 µM) IL-8 release, and expression of adhesion molecules CD11b/8), tumor necrosis factor alpha (TNF-α) production/such as reactive oxygen species, interleukin 8 (IL-

3.0) and a B solution (acetonitrile) at an initial flow rate of 2.3. HPLC analysis

Quantitative HPLC analysis of the main components was performed on an HPLC ELITE LaChrom system with a DAD detector and a Luna C18(2) column (250 × 4.6 mm, 5 µm) at 23 ºC, using a gradient elution. Separation of polyphenols was performed using a mobile phase consisting of an A solution (water acidified with phosphoric acid, pH

Reference substances were purchased as follows: quercetin, rutin, and caffeic acid were from Sigma Aldrich (Germany) and eupatorin, eupatilin, and β-ecdysone were from PhytoLab (Germany).

2.4. Cell lines and culture conditions

The following cell lines were purchased from the American Type Culture Collection (ATCC, USA) and used: NR8383 (normal rat alveolar macrophages), BT-20 (human mammary gland/ breast carcinoma), HepG2 (human hepatocellular carcinoma), Caco-2 (human colorectal adenocarcinoma), and Jurkat (leukemia peripheral blood lymphocytes). Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s modified Eagle’s medium F12 (DMEM-F12), Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), penicillin-streptomycin, Dulbecco’s phosphate-buffered saline (DPBS), and trypsin-EDTA solution were from Life Technologies (USA). For cell viability assay, 96-well microplates from Corning (USA) were used. Dimethyl sulfoxide (DMSO) and bacterial lipopolysaccharide (E. coli LPS K-235) were obtained from Sigma Aldrich (Germany). Cells were cultured in EMEM (BT-20, Caco-2), DMEM (HepG2), DMEM-F12 (NR8383), or RPMI 1640 (Jurkat) medium supplemented with 10% (Jurkat, Caco-2, HepG2), 15% (NR8383), or 20% (BT-20) FBS and penicillin-streptomycin (1%, 100 units/mL) and were maintained at 37 ºC in a humidified atmosphere with 5% CO₂. Media were replaced two to three times per week. Adherent cells were washed with DPBS (pH 7.0) and then treated with trypsin (0.05%)-EDTA (0.002%), collected, and centrifuged. Cells were aliquoted and 15 to 20 mL were transferred to 75-mL flasks, after which they were passaged every 5 days. Once a week, the nonadherent cell line (Jurkat) was established by centrifugation and subsequent resuspension at 1 × 10^6 viable cells/mL.

2.5. Survival of LPS-stimulated macrophages

An assay was used to assess the potential cytotoxic effect of the extracts on a normal rat macrophage cell line (NR8383) and also to evaluate their influence in the case of LPS stimulation. A total of 1 × 10^6 cells/well were seeded into 96-well plates and were allowed to settle for 24 h. The cells were then treated with several dilutions of extracts ranging from 25 to 150 µg/mL. Each concentration/assay was performed three times. A mixture of DMSO and water (extract solvent) was diluted with culture media in the same way as samples and served as a negative control. As positive control, levamisole (100 µg/mL) was added. The cells were incubated with the treatments for 24 h, after which 20 µL of MTS (5 mg/mL, CellTiter 96-Aqueous One Solution Cell Proliferation Assay, Promega) was added and further incubated for 4 h at 37 ºC. The absorbance was
measured at 492 nm with an LKB Chameleon microplate reader. Cell viability was expressed as a percentage of live treated cells compared with live control cells.

For evaluation of survival of LPS-stimulated macrophages, cells were treated with serum-free media supplemented with several dilutions of extracts (25–150 µg/mL) in the presence of LPS (10 µg/mL). LPS alone served as a negative control. After 18 h of incubation, the cultures were also subjected to MTS assay as described above.

2.6. Antiproliferative activity on cancer cells
An assay was used to assess the effect of the extracts on the survival of human cancer cell lines. A total of 1 × 10³ (BT-20, Caco-2, HepG2) or 2.5 × 10³ (Jurkat) cells/well were seeded into 96-well plates and were allowed to settle for 24 h. The cells were then treated with several dilutions of extracts ranging from 6.25 to 250 µg/mL. Each concentration/assay was performed three times. A mixture of DMSO and water (extract solvent) was diluted with culture media in the same way as samples and served as a negative control. As positive controls, several concentrations (6.25–100 µg/mL) of 5-fluorouracil were used. The cells were incubated with the treatments for 24–48 h, after which 20 µL of MTS (5 mg/mL, CellTiter 96-Aqueous One Solution Cell Proliferation Assay, Promega) was added and further incubated for 4 h at 37 °C. The absorbance was measured at 492 nm with an LKB Chameleon microplate reader. Cell viability was expressed as a percentage of live treated cells compared with live control cells.

2.7. Animal model of endotoxemia and survival test
All animal studies were approved by the Ethics Committee of the National Institute of Chemical-Pharmaceutical Research and Development. Briefly, NMRI mice (males, 6 weeks old, 20–25 g) purchased from the Cantacuzino Institute (Bucharest, Romania) were housed in a pathogen-free environment. Sepsis/lethal endotoxemia was induced by administration of bacterial endotoxin by intraperitoneal (IP) injection (20 mg/kg). At –48, –24, and +0.5 h post sepsis animals were administered IP saline (0.2 mL/20 g mouse) or EC/EA at indicated doses, and animal survival was monitored for up to 10 days. Mice were randomly assigned to the following groups: injection of saline (group I), injection of LPS (20 mg/kg) (group II), simultaneous IP injection of LPS (20 mg/kg) plus EC (250, 500, or 875 mg/kg) (groups III–V) or respectively EA (250, 500, or 875 mg/kg) (groups VI–VIII), or injection of levamisole (10 mL/kg) (group IX) for 3 days. Ten days after the last administration, animals were euthanized and blood was collected and analyzed.

In parallel similar experiments, mice were euthanized 18 h after LPS challenge to collect blood and assayed for plasma levels of cytokines.

2.8. Cytokine assays
The TNF-α, IL-1β, and IL-6 concentrations in the plasma samples were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits specific for mice (Novex-Life Technologies, USA) and following the manufacturer’s instructions. The results were recorded in terms of the optical density measured at 450 nm with an LKB Chameleon multiplate reader.

2.9. Statistical analysis
For the statistical analysis, the paired Student t-test was used. The results were expressed as means ± SD of at least three independent experiments. P < 0.05 was statistically significant. All analyses were performed with GraphPad Prism (version 7.03; GraphPad Software Inc., USA).

3. Results and discussions

3.1. HPLC analysis
Consecutive extraction of E. cannabinum raw material with chloroform and water, respectively, was conducted for separation of two corresponding fractions, EC and EA, with different profiles, correlated to the compounds’ polarity. As reference substances we selected ubiquitous phenolic compounds (rutin, caffeic acid, etc.) and methoxylated flavones mainly found in Asteraceae species. All selected compounds exhibit strong cytotoxic or antiinflammatory activity comparatively based on the used concentration. While EA lacks methoxylated flavones but contains high amounts of caffeic acid and rutin, EC is characterized by the presence of nonpolar compounds such as eupatorin, eupatilin, and quercetin. As for β-ecdysone, it is almost equally distributed in both fractions (Table).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference compound (mg/100 g extract)</th>
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<tbody>
<tr>
<td></td>
<td>β-Ecdysone</td>
</tr>
<tr>
<td>EC</td>
<td>10.05 ± 0.91</td>
</tr>
<tr>
<td>EA</td>
<td>11.78 ± 2.8</td>
</tr>
</tbody>
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*Values are means ± standard deviations of triplicate samples.
To ensure that the extracts were not toxic to normal cells, cell viability after treatment with various concentrations of EC and EA extracts was determined using a rat alveolar macrophage cell culture. The NR8383 cell viability decreased with increasing concentration of the extracts from 25 to 150 µg/mL (Figure 1). Generally, concentrations of 125 µg/mL yielded cell viability higher than 50%, whereas at 100 µg/mL it was higher than 80% in the case of both extracts.

In LPS-stimulated macrophages, both extracts failed to induce a protective effect. Comparing to LPS, for treated cells, the viability of cells treated simultaneously with extracts and LPS was lower. This could be explained by the fact that additional cytotoxic effects were induced by the extracts in LPS-stimulated cells (Figure 2).

3.2. Assessment of in vitro antiproliferative effect on tumor cells

We conducted a screening of several tumor cell lines’ viability after exposure to increasing concentrations of *E. cannabinum* polar and nonpolar extracts (6.25–250 µg/mL) for 24 and 48 h, as described in Section 2. The EC extract exhibited a dose-dependent inhibitory effect on all cell lines, with IC₅₀ of 7.35 µg/mL against Jurkat cells (Figure 3), 88.27 µg/mL against Caco-2 (Figure 4) cells, and over 100 µg/mL against HepG2 (Figure 5) and BT-20 cells (Figure 6) after 24 h of incubation. Jurkat cells were more susceptible to the inhibitory action of both extracts. EA had an effect only on this line, with an IC₅₀ of 13.77 µg/mL. The chloroform extract was more active than the aqueous one, which implies that active nonpolar principles are responsible for the antiproliferative activity in *E. cannabinaum*. Regarding the dynamics of inhibition, it was noted that after 48 h both extracts were acting differently. While EC inhibitory action was limited and after 48 h cells exhibited a recovery of proliferative activity (increased viability of Jurkat cells with 33.51% at maximum concentration of the extract compared to proliferation rate registered at 24 h), EA maintained its inhibitory action, decreasing cell viability by 14.63% (HepG2) and 24.21% (Jurkat) compared to the viability rate at 24 h. The same prolonged inhibitory effect was also shown by the reference compound, 5-FU (decreased viability of HepG2 cells by 46.63% and of Jurkat cells by 23.22% at maximum concentration tested compared to proliferation rate registered at 24 h).

Although the antiproliferative effect of phenolic compounds such as caffeic acid and rutin is well documented, in this study the EA extract containing high amounts of the two mentioned compounds failed to exert certain inhibitory action on the tumor cells tested. Previously, Elema et al. (1989) showed that the cytotoxic activity of *E. cannabinum* flavones is considerably lower compared to sesquiterpene lactones and consequently these compounds do not significantly contribute to the cytotoxic properties of this species. Castillo et al. (2015) worked on *E. illitum* and showed that flavonoids exhibit only moderate to low antiproliferative activities on human cancer cell lines A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), and T-47D (breast). Water-soluble compounds exhibit low antiproliferative effects towards HeLa, Eahy 926, and L929 cell lines.
compared to nonpolar or ethanol soluble compounds such as sesquiterpene lactones, as reported previously by Habtemariam and Macpherson (2000). In this regard, the *E. cannabinum* ethanolic extract had cytotoxic activity on HT29 cancer cells, leading to mitotic disruption and nonapoptotic cell death without severe induction of DNA damage, with synergistic effects with doxorubicin (Ribeiro-Varandas et al., 2014).

In addition, some methoxyflavones, including those found in our extracts, play key roles in exerting several pharmacological activities, including antiproliferative and cytotoxic effects. For instance, eupatilin (5,7-dihydroxy-3',4',6-trimethoxyflavone) exhibits antioxidant, antiinflammatory, antiallergic, and neuroprotective activities (Du et al., 2017); induces apoptosis in both human promyelocytic leukemia HL-60 cells (Seo and Surh, 2001) and human gastric cancer AGS cells (Kim et al., 2005); and inhibits LPS-induced expression of inflammatory mediators (iNOS, TNF-α, IL-1β, IL-6, COX-2, and MCP-1) in macrophages by suppression of NF-κB activation (Choi et al., 2011). Eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone) is a potent antiproliferative agent against several cancer cell lines such as organotypic 3D prostate cancer (Salmela et al., 2012), human cervical adenocarcinoma (HeLa), human gastric adenocarcinoma (MK-1), murine melanoma (B16F10), murine colon carcinoma (26-L5), or human breast cancer cell line (MDA-MB-468), but it has no cytostatic effects in the

**Figure 3.** Effect of EC and EA extracts on viability of Jurkat cells after 24 h (a) and 48 h (b) of exposure. Values represent mean ± SD (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).
Figure 4. Effect of EC and EA extracts on viability of Caco-2 cells after 24 h of exposure. Values represent mean ± SD (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

Figure 5. Effect of EC and EA extracts on viability of HepG2 cells after 24 h (a) and 48 h (b) of exposure. Values represent mean ± SD (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).
normal human breast cell line (MCF-10A) (Dolečková et al., 2012). The outcome of this study proves that these compounds might be responsible for the antiproliferative effect, since the EC extract, the most specifically active one, contains the abovementioned methoxyflavones.

3.3. Effect of *E. cannabinum* extracts on survival times in a mouse model of endotoxemia

LPS has been used in various studies because of its ability to induce a rapid response at the host level in a dose-dependent manner. LPS induces cytokine secretion (TNF, IL-1, IL-6) mediated by TLR-4 receptors. The highest rates of activated leukocytes were observed upon the administration of the largest LPS doses (20 mg/kg) (Burkovskiy et al., 2013). However, in the previous in vitro assay, assessment of cell viability by the MTS test showed that both extracts failed to protect LPS-stimulated macrophages. Though LPS stimulation involves other cells, the effects of EC and EA on the survival times in a mouse model of endotoxemia were evaluated and are shown in Figures 7 and 8. The extracts were administrated as chemopreventive agents at 48 and 24 h before LPS challenge and at 30 min after it to induce protection against septic shock.

Survival rates were similar for minimum and maximum doses tested for both extracts. The first 24 h were obviously dose-dependent for both extracts and a difference can be noted between them: at 500 mg/kg extract concentration, EC induced 100% protection, while EA decreased survival rate up to 80%. For all extracts and LPS, the maximum decrease in survival rate was registered at 24 h and it remained constant throughout the monitoring period. Levamisole, the reference compound, induced 100% protection.

3.4. Effects of *E. cannabinum* extracts on cytokine production in a mouse model of endotoxemia

TNF-α, IL-1β, and IL-6 are key proinflammatory cytokines induced by LPS. IL-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis. An immediate and transient expression of IL-6 is generated in response to environmental stress factors such as infections and tissue injuries (Tanaka et al., 2014).

The effect of EC and EA on the dynamics of IL-6 in plasma of NMRI mice is shown in Figure 9. Maximum doses tested of EC and EA (500 and 875 mg/kg) decreased IL-6 levels at 18 h after LPS challenge (P < 0.01), with higher impact of EA (87% decrease compared to LPS alone). At the minimum dose tested, 250 mg/kg, EC protected it by 40% while EA only 16.8%. The reference compound, levamisole (10 mg/kg), showed 98% inhibitory effect on IL-6 level (P < 0.001) compared to LPS alone.

As for the inhibitory capacity of *Eupatorium* extracts on IL-1β levels, the EA at high concentrations (500 and 875 mg/kg, P < 0.01) decreased its expression by 60%, similarly to levamisole (Figure 10). EC induced a significant protection of 77%–100% at all concentrations tested, while EA induced a decrease of TNF-α level of 45.84%–100%. Levamisole decreased TNF-α expression up to 30%.
Significant (for IL-1α and IL-1β) and moderate (for TNF-α) downregulation of cytokines after LPS challenge was reported for methanol, ethanol, and dichloromethane extracts of *E. perfoliatum* having eupafolin and a dimeric guaianolide as probable responsible compounds (Maas et al., 2011), the sesquiterpene fraction of *E. lindleyanum* (Chu et al., 2016), and 2-hydroxyeupatolide, a sesquiterpene lactone, widely found in the genus *Eupatorium* (Ke et al., 2017).

IL-6, TNF-α, and IL-1β are under transcription control of NF-κB, which is activated by LPS and plays significant roles in proliferation, cell survival, and inflammation.
In this study, EA had a greater inhibitory effect on proinflammatory cytokine expression than EC; it might be suggested that the hydrosoluble compounds are involved in inflammation-activation pathways.

3.5. Conclusions

This study aimed to prove some of the beneficial effects of *E. cannabinum* species, widely used based on its therapeutic properties. Testing the influence of EC and EA extracts against proliferation of BT-20, HepG2, Caco-2, and Jurkat cancer cell lines could not highlight the responsible compounds for this effect. While the nonpolar extract, rich in methoxylated flavones, had a moderate cytotoxic effect after 24 h of exposure on most of the cell lines tested, the polar extract, with a high content of caffeic acid and rutin, exhibited a certain antiproliferative effect mostly after 48 h of exposure.
The effects of two *E. cannabinum* extracts with different solubilities were tested also on an endotoxin-induced mouse model of endotoxemia. Although the LPS-stimulated rat alveolar macrophages did not show any protective effect in vitro, the results of in vivo studies demonstrated that preventive administration of EC and EA increased the survival times of mice and led to inhibition of proinflammatory cytokines (TNFα, IL-1β, and IL-6).

This study confirmed the potential cytotoxicity of *E. cannabinum* as well as the protective effect against bacterial endotoxins and proinflammatory factors. Both polar and nonpolar compounds are involved in exerting these effects, but further analytical studies are needed to identify the key responsible compounds and the biochemical pathways for their action.

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**References**


