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Article

Antimicrobial and Immunomodulatory Activities of Dried Extracts of *Echinacea Purpurea*

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The use of *Echinacea purpurea* (EP), a plant native from North America, is widely diffused throughout the world, presenting many pharmacological applications, mainly for the treatment of infections of respiratory and urinary tracts. Due to the widespread commercialization of EP-based products, an effective evaluation of their pharmacological properties is essential to assure efficacy during clinical use. In this study, *in vitro* tests were performed to evaluate the antimicrobial activity of dried extracts of EP by the microdilution method. In addition, a phagocytosis model was employed to assess the immunomodulatory potential of the extracts. The increase in reactive oxygen species production, as well as the intracellular proliferation rate of *Cryptococcus gatti* after phagocytosis by macrophages in the presence of EP dried extracts were also evaluated. The analyzed samples showed no significant antibacterial activity; however, a slight antifungal activity was verified. Positive effects of EP extracts on the modulation of cellular immune response were observed in different experiments, indicating that this mechanism may contribute to the control and treatment of infections.

Keywords: *Echinacea purpurea.* Antimicrobial activity. Immunomodulatory activity. Cafeic acid derivatives.

INTRODUCTION

Echinacea purpurea (EP), belonging to Asteraceae family, is a plant native from North America, also known as purple coneflower (Barnes *et al*., 2005). Traditionally, the aerial parts, roots and eventually the entire plant are used for medicinal purposes, mainly to treat infections in respiratory and urinary tracts (Ardjomand-Woelkart, Bauer, 2016). Several compounds from different classes have been isolated and identified in EP extracts. Alkamides, caffeic acid derivatives and polysaccharides are the three main groups of secondary metabolites found in the plant. Among the polyphenols derived from caffeic acid, the main compounds are caftaric acid, chicoric acid and chlorogenic acid (Manayi, Vazirian, Saeidnia, 2015).

Considering the significant increase of bacterial resistance in hospitals and communities, the search for new antimicrobial agents or even adjuvants in the treatments of infections is essential. The number of studies relating the use of natural products with antimicrobial or immunomodulatory activities has significantly increased in recent years (Gyawali, Ibrahim, 2014; Moloney, 2016). Previous works demonstrated that EP could reduce the proliferation of bacteria related mainly to respiratory infections, such as *Streptococcus pyogenes*, *Legionella pneumophila*, *Haemophilus influenza* and *Clostridium difficile* (Sharma *et al*., 2010; Hudson, 2012). Antifungal activity of EP extracts was also reported against *Candida albicans* and *Saccharomyces cerevisiae* (Barnes *et al*.,

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2005; Manayi, Vazirian, Saeidnia, 2015). Furthermore, EP activity against microorganisms may be related to the enhancement of innate immunity by the plant. The mechanism by which EP modulate the cellular immune response is not entirely elucidated and may be related to activation of neutrophils, macrophages and natural killer cells (Manayi, Vazirian, Saeidnia, 2015; Tsai *et al*., 2012), stimulation of phagocytosis and production of pro-inflammatory mediators (Hudson, 2012; Zhai *et al*., 2009) or increase in production of reactive oxygen species (Fonseca *et al*., 2014; Stanisavljevi *et al*., 2009). Although these well-established activities for EP, it is unclear if the commercial products based on this plant, such as dried extracts, have exactly the same properties, mainly due to extraction process, employed solvents and storage conditions.

The wide commercialization of herbal products based on EP evidences the need to evaluate these products regarding the pharmacological activity. Hence, this study aimed to assess the antimicrobial activity and the immunomodulatory potential of different commercial EP dried extracts.

MATERIAL AND METHODS

Chemicals and Reagents

Dried extracts samples of *Echinacea purpurea*, usually employed as raw material for capsule production, were kindly donated by compounding pharmacies. Ultra-pure water was obtained from a Millipore® system. Itraconazole, chloramphenicol and chlorogenic acid reference standards were purchased from Sigma-Aldrich®. All other reagents used in the study presented analytical grades.

Antimicrobial assay

The susceptibility of microorganisms to EP dried extracts was determined according to broth microdilution method, presented as a consensus standard by Clinical and Laboratorial Standards Institute (CLSI M27–A2, 2008; CLSI M38-A2, 2008; CLSI M07-A9, 2012). Antibacterial activity was evaluated against six Gram-negative Bacteria (*Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica Typhimurium* ATCC14028, *Shigella flexnelli* ATCC 12022, *Acinetobacter baummani* ATCC19606 and *Klebsiella pneumoniae* ATCC 13882) and two Gram-positive bacteria (*Staphylococcus aureus* ATCC 33591 and *Enterococcus faecalis* ATCC 19433). Antifungal activity was tested against *Cryptococcus gattii* R265, *Cryptococcus gattii* ATCC 24065, *Cryptococcus neoformans* H99, *Cryptococcus neoformans* ATCC 24067, *Candida albicans* ATCC 18804, *Aspergillus fumigatus* (clinical strain), *Trichophyton inderdigitale* ATCC 9533, *Trichophyton rubrum* ATCC 28189 and *Microsporum gypseum* (clinical strain). The inocula of the tested strains were prepared from grown cultures (according to the tested microorganism). The suspensions were prepared in phosphate buffered saline (PBS) to obtain final concentrations of 1.0 – 2.0 x 10^5 CFU/mL for bacteria, 1.0 - 5.0 x 10^3 CFU/mL for yeasts and 2.0 – 4.0 x 10^4 CFU/mL for filamentous fungi.

Stock solutions of EP dried extracts were prepared in water at 10 mg/mL. Serial twofold dilutions were performed in Roswell Park Memorial Institute (RPMI) medium to achieve the final concentrations: 512, 256, 128, 64 and 32 μg/mL. A standard solution of chlorogenic acid at 512 μg/mL was also evaluated for comparison. Then, 100 μL of each solution was transferred to a sterile 96-well microplate with 100 μL of adequate media (Müeller-Hinton for bacteria, Sabouraud dextrose for yeasts and potato dextrose for filamentous fungi). An aliquot of 100 μL of each bacterial or fungi suspension was added to the microplate, in duplicate. Negative and positive controls were used for each tested strain. The plates were incubated at 35 °C for 24 h (bacteria) or 72 h (fungi), and 28 °C for 7 days for *Trichophyton* spp. Minimum inhibitory concentration (MIC) was determined by visual inspection as the lower concentration of the extracts that inhibited the microbial growth for each strain.

Immunomodulatory assays

For the immunomodulation tests, *in vitro* tests were initially performed to evaluate the potential of EP dried extracts in stimulating the ability of macrophages

to phagocytosis *Cryptococcus gattii* strain L27/01. The increase in reactive oxygen species (ROS) production, as well as the intracellular proliferation rate of *Cryptococcus gatti* after phagocytosis by macrophages in the presence of EP dried extracts were also evaluated.

Briefly, bone marrow cells recovered from mice femurs and tibias were counted using a hemocytometer and the concentration was adjusted to 2×10^6 cells/mL for incubation in BMM medium (RPMI supplemented with 30% L929 growth conditioning media, 20% bovine fetal serum (Gibco), 2 mM glutamine (Sigma-Aldrich), 25 mM HEPES pH 7.2, 100 units/mL of penicillin-streptomycin (Life Technologies). Fresh media were added every 48 h. Bone marrow-derived macrophages (BMDM) were collected on day 7 and used for subsequent experiments. Sterile circle coverslips (13-16 mm) were introduced in the bottom of each plate well of 24-well plates of phagocytosis. Then, the cell concentration was adjusted to 2.0 x 10^5 cell/mL and 500 µL was added to 24 well plate to phagocytosis and killing assay and 100 uL to 96 well plate to ROS quantification. EP dried extracts solutions at two concentrations (10 and 100 µg/mL) were added and the plates were incubated at 37 °C with 5% CO_2 for 30 min before infection. Viable yeasts of *C. gatti* were adjusted to 0.4 x 10^5 cell/mL (1:5 ratio of yeasts to macrophages) and added to macrophage culture on the plates. The protocol of the study was previously approved by Ethics Committee on Use of Animals of Universidade Federal de Minas Gerais (protocol number 34/2016). After 24 h, coverlips from 24 well plate from phagocytosis assay were carefully removed, washed with sterile PBS, fixed with ice methanol and stained with Panoptic dye. Macrophages were counted using optical microscopy and phagocytic capacity was expressed by the percentage of macrophages with internalized fungi. To investigate the capability of macrophages to control fungal proliferation after phagocytosis, at 24 h after infection, each well was washed with PBS and cells were lysed using 200 µL of sterile distilled water. Each plate was incubated at 37 ° C for 30 min for complete cell lysis. Then, 50 µL of the lysate was collected and plated on Sabouraud dextrose agar to determine the number of colony forming units (CFU). The intracellular proliferation rate was calculated by dividing CFU at 24 hours (*t* 24h) by the initial CFU at 3 hours (*t* 3h) (Ribeiro *et al.*, 2017).

For the determination of reactive oxygen species, the same concentrations EP dried extracts concentrations were tested. After treatment regimens and infection, cells were incubated at 37 °C with 5% CO_2 for 3 and 24 h, in the presence of 10 µM of dichlorofluorescein diacetate (DFCDA; ThermoFisher). The response was determined in a fluorimeter, using excitation and emission wavelengths at 500 nm, and expressed as arbitrary units of fluorescence (Ribeiro *et al.*, 2017).

Quantitation of caffeic acid derivatives in EP dried extracts

The content of caffeic acid derivatives in each sample of EP dried extract was determined by UPLC-DAD in triplicate (Oliveira *et al.*, 2021). The analysis was carried out on a Zorbax Eclipse Plus C_{18} column (2.1 x 50 mm; 1.8 µm), at 30 °C and mobile phase composed of acetonitrile and 0.05% aqueous formic acid (10:90), at a flow rate of 0.2 mL/min. UV detection was performed at 300 and 330 nm. Concentrations of caftaric, chicoric and chlorogenic acids were determined and the final result was expressed as weight of caffeic acid derivatives by weight of dried extract percentage (w/w %).

Statistical Analyses

The results were statistically compared using GraphPad Prism® Statistical Software 5.0.

RESULTS

Antimicrobial assay

The evaluation of the antimicrobial activity of EP dried extracts and chlorogenic acid reference standard regarding different strains of microorganisms indicated that the extracts could not inhibit the growth of any tested strain of bacteria. A slight antifungal activity was evidenced, mainly regarding *C. neoformans* and *C. gattii*. As demonstrated in Table I, samples 1, 2, 4 and 5 did not inhibit fungal growth at the tested concentrations. On the other hand, sample 3 presented MIC = 512 µg/mL for all *Cryptococcus sp.* strains.

TABLE I - Determination of MIC for EP dried extracts regarding different fungal strains

Microorganisms	EP dried extract					
	1	2	3	4	5	CA
C. gattii R265	>512	>512	512	>512	>512	512
C. gattii ATCC 24065	>512	>512	512	>512	>512	256
C. neoformans H99	>512	>512	512	>512	>512	512
C. neoformans ATCC 24067	>512	>512	512	>512	>512	512
Trichophyton inderdigitale ATCC 9533	> 512	> 512	> 512	> 512	> 512	512
Trichophyton rubrum ATCC 40051	> 512	> 512	> 512	> 512	> 512	512
Microsporum gypseum	> 512	> 512	> 512	> 512	> 512	512

CA = Chlorogenic acid

Immunomodulatory assays

Phagocytosis index was calculated as the percentage of macrophages with internalized fungi, regarding total macrophage number. Results were evaluated after 24 h incubation, with four EP dried extract samples (E1 to E4) at 10 and 100 µg/mL (Figure 1A). The higher dose tested led to increased phagocytosis, with a more prominent effect for extracts 3 and 4. The results from the ROS production assay were expressed as fluorescence arbitrary units (Figure 1B). Comparing the infected macrophage group with treated groups, it can be observed that EP dried extracts stimulate ROS production in a dose-dependent way. All extracts, at the dose of 100 µg/mL, were able to increase the ROS production by macrophages, and extract 3 was responsible for the higher ROS increase. The intracellular proliferation rate of *C. gattii* revealed that, although there is a difference in ROS production according to the dose tested, it did not reflect the ability of macrophage in killing the fungus in this assay, since at 100 µg/mL there was increased fungal proliferation for extracts 2 and 4. On the other hand, lower fungal growth inside macrophages was evidenced by extract 3 (Figure 1C).

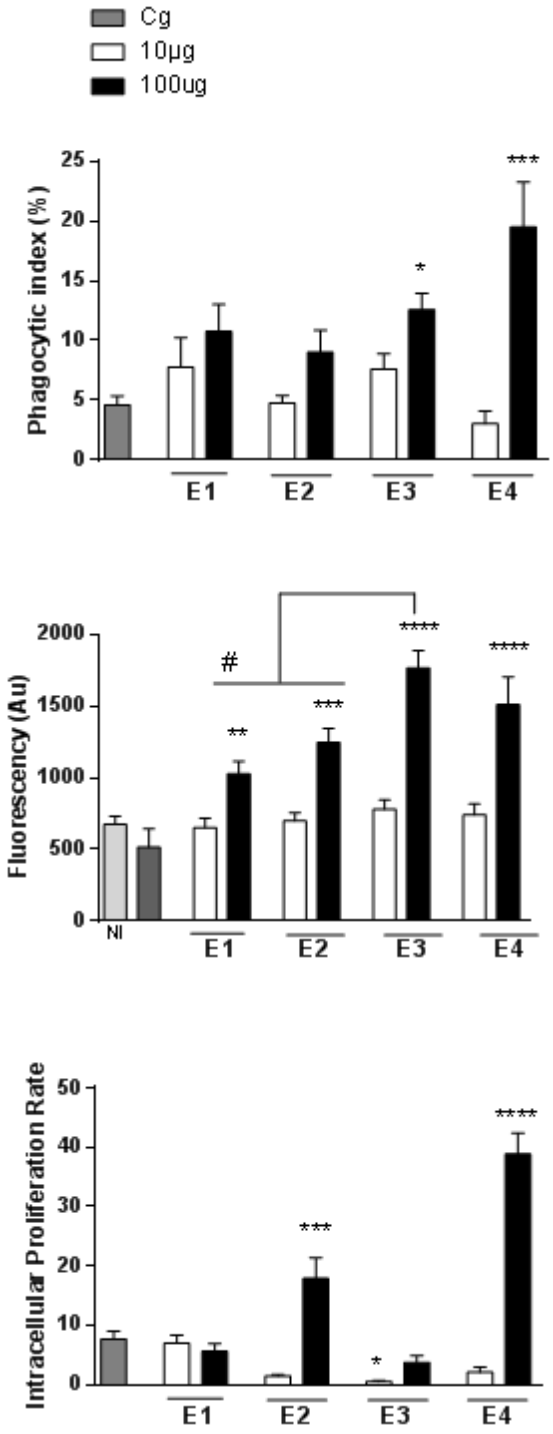

FIGURE 1 - Fungicidal activity from macrophage EP treated against *Cryptococcus gattii*. (A) Phagocytic index and (B) ROS production by these cells were analyzed 24 hours post infection of cells treated or not with EP dried extract samples at 10 and 100 µg/mL. (C) Fungal intracellular proliferation rate (IPR) was obtained by ratio 24 h/3 h fungal recovery. Bars represent the mean ±SD. *p: were significantly different ($p < 0.05$), *** ($p<0.001$), **** ($p<0.0001$) from Cg infected non treated. # ($p<0.05$ comparing E2 and E1 *vs* E3). BMDM (Cg). NI: non-infected.

Quantitation of caffeic acid derivatives in EP dried extracts

The individual and total contents of caffeic acid derivatives in the samples of EP dried extract are presented in Table II. It was observed a similar amount of caffeic acid derivatives in the extracts, between 0.18 and 0.26% (w/w), except for sample 3, which demonstrated a high amount of these compounds (0.71% w/w). In addition, sample 3 presented the highest content of caftaric acid (0.51% w/w) and chicoric acid (0.18% w/w). According to the quality reports of suppliers, extract 3 was prepared using the roots of *Echinacea purpurea*. In contrast, all other extracts were obtained from aerial parts or aerial parts mixed with roots and stems of the plant, and this difference may be related to the variation in caffeic acid derivatives.

TABLE II - Content of caffeic acid derivatives in EP dried extracts

Sample	Caftaric Acid % (w/w)	Chlorogenic Acid % (w/w)	Chicoric Acid % (w/w)	Total content % (w/w)
1	0.11	0.05	0.04	0.20
2	0.11	0.08	0.04	0.23
3	0.51	0.02	0.18	0.71
4	0.11	0.02	0.05	0.18
5	0.11	0.01	0.13	0.25

DISCUSSION

Traditionally, *Echinacea purpurea* is used for the treatment of respiratory and urinary infections. Sharma *et al.* (2010) demonstrated a significant antibacterial activity of an EP ethanolic extract obtained with aerial parts and roots against respiratory bacteria. The authors attributed the observed activity to a bactericidal effect of the extract and an anti-inflammatory effect, which could reverse the inflammation caused by these bacteria. On the other hand, samples of EP dried extracts evaluated in the present work did not demonstrate activity against bacteria strains and,

as previously mentioned, lack of biological activity may be related to variations on the part of the plant, extraction methods and solvents, or even low contents of vegetal markers in assayed extracts. Many biological activities were previously reported for caffeic acid derivatives, such as chlorogenic acid, including antimicrobial activity (Zheng *et al.*, 2016; De Vita *et al.*, 2014). Moreover, Kong *et al.* (2017) demonstrated that chlorogenic acid decreased biofilm formation by *Aspergillus fumigatus*. These reports are consistent with the observed antifungal activity of chlorogenic acid, although this compound did not demonstrate antibacterial activity. Chlorogenic acid presented MIC = 512 μg/mL for six tested fungal strains and MIC = 256 μg/mL for *C. gattii* ATCC 24065.

The results obtained in our biological tests may indicate that a possible way by which *Echinacea purpurea* is effective for the control of infections is an indirect mechanism. The direct antimicrobial effect appears to be less pronounced, since no significant antibacterial activity was observed and only a slight antifungal activity was verified against the strains evaluated under the conditions of the tests. It is fundamental to develop a better understanding of the antimicrobial mechanism of plant crude extracts on pathogenic microorganisms. The mechanism could involve direct inhibitory action or the extract can induce important immunological alterations during pathogen host interaction.

Macrophages are important innate immune cells that play a key role in host defense and homeostasis and their phenotype and function that are regulated by the signals from their environment. The data revealed variability in the effects among EP extracts, which may be partially explained by the differential concentration of individual and total caffeic acid derivatives. It was verified that EP dried extracts stimulated phagocytosis of the fungus by the macrophages and increased the production of reactive oxygen species by macrophages infected by *C. gatti*, in a dose-dependent way. Extract 3 presented the higher concentration of caftaric acid, chicoric acid and total caffeic acid derivatives and higher ROS production induction. This relation was not observed for the other samples, indicating that this biological activity may also be related to other compounds of EP extracts, such as alkamides or polysaccharides (Manayi, Vazirian, Saeidnia, 2015). All extract samples at 100 μg/mL showed a statistical difference for ROS production compared with infected macrophages in the absence of EP extracts. Since ROS production contributes to the resolution of infections, as part of a defense mechanism against many pathogens (Aguirre, Hansberg, Navarro, 2006), a considerable potential of EP dried extracts as an adjuvant in the modulation of the immune response was verified. Our results are consistent with Roesler *et al.* (1991), which demonstrated that purified polysaccharide extracts from *Echinacea purpurea* increased the production of reactive oxygen intermediates and inhibited the growth of fungi *in vivo.*

In addition, the antioxidant activity of caffeic acid derivatives is well established and has been previously reported (Sidoryk *et al.*, 2018). Notably, the antioxidant potential of *Echinacea purpurea* was evaluated by Aarland *et al.* (2017), which demonstrated the correlation between the high content of phenols and total flavonoids with the higher antioxidant activity of the extracts. Dalby-Brown *et al.* (2005) found a synergistic effect when testing chicoric acid or echinacoside combined with alkamides or aqueous extract of *Echinacea purpurea,* by means of *in vitro* antioxidant assays.

It was observed no increase in ROS production in the infected untreated cells compared to control (not infected) cells. The main virulence factor of *Cryptococcus gattii* and *Cryptococcus neoformans* is the polysaccharide capsule and the antioxidant activity of this capsule is well established. In a previous study of our research group (Santos *et al.*, 2014), the same tested strain (*Cryptococcus gattii* L27/01) demonstrated the ability to neutralize ROS produced by macrophages, related to the fungal polysaccharide capsule.

The functional tests showed that EP enhanced the phagocytic activity of macrophages against *C. gatti*. However, only extract 3 at 10 μg/mL successfully controlled the intracellular proliferation rate of the fungi. An opposite effect was observed for extracts 2 and 4 at 100 μg/mL, which seem to have inhibited the fungicidal activity of macrophages. There is a probable modulation of mediators produced by macrophages in the presence of different concentrations of extracts. Additional studies are still needed to investigate the molecular mechanisms by which these events occur.

In conclusion, although no significant activity against bacteria strains was observed, EP dried extracts positively affected the modulation of cellular immune response in different ways, contributing to the control and treatment of infections. The obtained results indicate an immunomodulatory potential that should be better investigated in further studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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