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Attenuation of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice by Licochalcone A

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Abstract. Multiple sclerosis (MS) is an autoimmune inflammatory and demyelinating disease of the central nervous system (CNS) that affects more than 2.5 million people worldwide. The experimental autoimmune encephalomyelitis (EAE) is an appropriate and a well-establish model for studying the pathogenesis of MS. Licochalcone A (LicoA) is a chalcone obtained from the roots of *Glycyrrhizainflata* (Fabaceae) that has *in vitro* immunomodulatory effects. EAE was induced in C57BL/6 mice with myelin oligodendrocyte glycoprotein and we have investigated the treatment of LicoA in this animal model. LicoA was isolated from *G. inflata* and was orally administered during the development of EAE. The capacity of absorption and distribution of LicoA, after gavage, to the brain was performed by HPLC. The clinical course and body weight were performed daily, cytokines (ELISA) and oxygen radicals production (NO and H_2O_2) were investigated. The CNS sections were stained by hematoxylin and eosin. After the treatment, by HPLC, at the first time, we analyzed the penetration between tissue/plasma, and our results showed that LicoA was present in serum and reached the mice brain with a good distribution. LicoA reduced clinical score and severity of EAE-mice, as well as inhibited H_2O_2, NO, TNF-α, IFN-γ and, mainly, IL-17 production. Histopathological analysis confirmed that LicoA treatment significantly reduced the numbers of inflammatory infiltrates and attenuates neurological damages in the CNS. These findings demonstrate that the oral treatment of LicoA significantly ameliorated the inflammatory signs associated with EAE, since it is effective at reducing both disease onset and severity.
Keywords: Licochalcone A, multiple sclerosis, cytokines, histopathology, experimental autoimmune encephalomyelitis.

Introduction

Multiple sclerosis (MS) is an autoimmune and chronic disease of the central nervous system (CNS) characterized by demyelination, glial scar formation and subsequent degeneration of axonal and neuronal damage which results in a progressive neurological function deficit (Alipour et al., 2024; Bjelobaba et al., 2018; Kakalacheva & Lünemann, 2011; Lassmann, 2011). This disease has limited treatment options and a high socio-economic impact that affects more than 2.5 million people worldwide and can affect people of all classes, race and age, however it is known that the highest prevalence occurs in young adults (Chen et al., 2010; Fontes et al., 2014; Gheidari et al., 2024). Experimental autoimmune encephalomyelitis (EAE) is the most commonly, useful and exploited model for studying MS, imitating several histopathological aspects and immunological facets of MS (Bjelobaba et al., 2018; McGinley et al., 2018).

It is known that the immunopathogenesis of both EAE and MS is immune-mediated mainly by Th1 and Th17 cells and defensive responses by Th2 and Treg cells. If Th1 cells are stimulated, some cytokines are produced, such as interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). On the other hand, Th17 cells activate mainly the production of IL-17, while both cells (Th1 and Th17) induce the production of oxygen radicals, such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) (Rodgers & Miller, 2012). Several studies showed that infiltrating immune cells produce nitric oxide (NO), reactive oxygen species (ROS), chemokines and pro-inflammatory cytokines, such as IFN-γ,TNF-

α and IL-17 that are associated with inflammation and impaired neural functions (Cheng et al., 2017; F. Chu et al., 2018; Giacoppo et al., 2015; McGinley et al., 2018). Oxidative stress, with high production of NO and H_2O_2, in CNS is also pathogenic, leading to neurological damage and neuroinflammation (F. Chu et al., 2018; Giacoppo et al., 2015; McGinley et al., 2018).

However, the etiology of MS is not completely elucidated, and currently its treatment has a high cost, several drawbacks as limited efficacy and many adverse effects (Corrêa et al., 2010; Giacoppo et al., 2015). Therefore, the development of more effective and well tolerated drugs in the treatment of MS are important to improve benefits in this treatment and to promoting slow disease progression (Chen et al., 2010; Fontes et al., 2017; Olejnik et al., 2024).

Plants are one of the classic forms of alternative therapy to the population, and knowledge about their action designates, in many cases, the only therapeutic resource of many communities and ethnic groups (Manzano et al., 2016). At this juncture, natural products have played a central role in the development of new drugs more effectives and tolerable in inflammatory diseases (Newman & Cragg, 2012). So far, several natural compounds, which were isolated from medicinal plants, prove to be having potent immunomodulatory activity (Filho et al., 2004) and potential benefits of phytochemicals from herbs and plant extracts are searched (Rasool et al., 2014).

Licochalcone A (LicoA) is a natural chalcone found mainly in *Glycyrrhiza* species, such as *G. glabra*(Barfod et al., 2002) and G. inflate (D. Liu et al., 2018). Recently, many studies have shown that LicoA has several biological activities, among them anti-cancer, anti-inflammatory, antioxidant and antimicrobial activities (D. Liu et al., 2018; M. Liu et al., 2024; Shen et al., 2024). Kim et al. (Kim et al., 2012) have demonstrated that chalcones were able to attenuate the inflammatory responses induced by LPS in microglial BV1 cells and protect dopaminergic SH-SY5Y cells from the cytotoxicity of 6-hydroxydopamine. Recent studies have also shown that levels of TNF-α, IL-6 and IL-1β in serum and levels of MDA and ROS have been reduced. These same studies showed that LicoA was also able to reverse depletion of GSH and SOD, indicating that treatment with LicoA attenuated the inflammatory response and oxidative damage in the liver of C57Bl / 6 mice (Lv et al., 2019). In addition, LicoA was able to inhibit the production of TNF-α, IFN-γ and IL-17, which are inflammatory mediators present in the immunopathogenesis of MS and in its animal model, EAE (X. Chu et al., 2012). The ability of LicoA to reduce the inducibility of nitric oxide synthase (*iNOS*) (Furusawa et al., 2009) and to inhibit the activation of Stat-3 (transcription factor for Th17 differentiation) (Funakoshi-Tago et al., 2008). In this regard, we report that LicoA possesses immunomodulatory activity *in vitro* in animal spleen cells induced by EAE (Fontes et al., 2014).

Thus, as part of our works on EAE and biological activity on natural products and phytochemical compounds (Dias et al., 2014; Fontes et al., 2014, 2017)we did, at the first time the quantification of LicoA in serum and brain after oral administration. After that, the main objective were to investigate the *in vivo* activity of LicoA in C57BL / 6 mice induced with EAE.

Material and Methods

Isolation and purification of LicoA

Dried extract of *G. inflata* was provided by Shanghai Openchem International CO., Ltd (Shanghai, China). The dried extract of *G. inflata* (10 g) was chromatographed over silica gel (60H, 100-200 mesh ASTM, Merck) under vacuum-liquid chromatography system (glass columns with 5-10 cm i.d), using dichloromethane (DCM)-methanol (MeOH) mixtures in increasing proportions to afford eight fractions. The resulting fraction 3 (DCM: MeOH 7:3 v/v; 1,7 g) was submitted to flash column chromatography (450 x 25 mm glass column, 5 mL/min) over silica gel (230-400 mesh, Merck), using DCM-MeOH (95:5 v/v) as mobile, furnishing 0.5 g of LicoA. The chemical structure of LicoA was established by ^{1}H- and ^{13}C-NMR (Nuclear Magnetic Resonance) data analysis (Brucker ARX 300 spectrometer) and by comparison of the data with literature(Fontes et al., 2014). Purity of LicoA was estimated to be higher than 95% by both ^{13}C-NMR and HPLC analysis using different solvent systems (Figure 1).

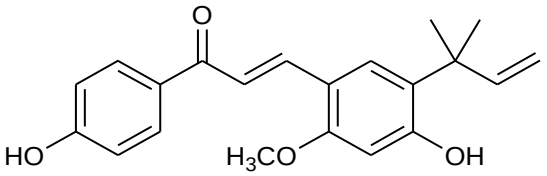

Figure 1. Chemical structure of licochalcone A (LicoA) isolated from the roots extract of *G. inflata*.

Animals

Female C57BL/6 mice (21-23g; 8-12 weeks old) were obtained from the animal care facilities at the Federal University of Juiz de Fora (CBR/UFJF) and maintained in micro isolator cages. All animal care and experimental protocols were approved by the Ethical Committee for Animal Care of the Federal University of Juiz de Fora (Protocol n^{o}. 039/2010).

HPLC detection and quantification of LicoA in mouse serum and brain

LicoA was administered (15mg/kg) by gavage in mice (group of five). One hour after, they were anesthetized (ketamine and xylazine 90mg:10mg/kg) for blood collection by cardiac puncture and then sacrificed by decapitation. Blood samples were centrifuged (10000 rpm, 10 min., 25° C) and 100µL of serum from each mouse were added with 200µL acetonitrile to precipitate proteins. The samples were homogenized and centrifuged and the supernatants were filtered with 0.25 µm

filters and injected into the chromatographic system. After decapitation, brain tissues were removed immediately, washed in ice cold saline, dried, weighed precisely, and macerated. Brain tissues (100 mg) were added with 200uL of acetonitrile, the homogenates were centrifuged, and supernatant was filtered with 0.25 µm filters and injected into the chromatographic system.

Quantification of LicoA was determined by HPLC-UV (Nadelmann et al., 1997). The method was developed in a Waters system equipped with an Alliance e2695 Separation Module, quaternary pump, autosampler, degasser, column heater and double channel UV-Vis detector (Milford, USA). The Empower 3 software was used for system control, peak integration and data analysis. Separation was carried out in a X-Bridge C_{18} column (150 x 4.6 mm, 5µm; Waters, Milford, USA) attached to a C_{18} guard cartridge (20 x 4.6 mm, 5µm; Waters, Milford, USA). The gradient mobile phase was composed by water acidified with ortho-phosphoric acid 0.1% (A) and methanol (B). The pump was programmed as follows (A:B): 0-5 min 50:50, 5-10 min 30:70, 10-12 min 20:80 and 12-15 min 50:50, (total gradient time: 15 min), at a flow rate of 1.1 mL/min, injection volume of 30 µL, column heater at 35°C and the UV detector set at 372 nm.

Induction of EAE

Mice were subcutaneously (s.c.) immunized at the tail base with 100 µg of $MOG_{35–55}$ (Sigma Chemical Co., Saint Louis, EUA), emulsified with complete Freund's adjuvant (CFA) (v/v) (Sigma Chemical Co., Saint Louis, EUA), and supplemented with 400 µg of attenuated *Mycobacterium tuberculosis* H37RA (Difco, Detroit, USA). *Pertussis toxin* 300 ng/animal (Sigma Chemical Co., Saint Louis, EUA) was injected intraperitoneally (i.p.) on the day of immunization and 48 h later (Alves et al., 2012). Non-immunized mice were used as control.

In vivo treatment with LicoA after induction of EAE

Mice were immunized and divided into four groups (n = 6): negative control group (CN), which non-immunized mice received only vehicle (5% tween 80 in saline); $MOG_{35–55}$ immunized group that received no treatment (EAE group); And $MOG_{35–55}$ immunized groups that were orally treated with LicoA at doses of 15 mg/kg/day (L15 group) and 30 mg/kg/day (L30 group). These doses of LicoA were based on previous studies that showed its *in-vivo* anti-inflammatory effects (Furusawa et al., 2009). All *in vivo* treatments were performed daily, per gavage, for 9 days, as from in the 10th day post-immunization, when 80% of the animals showed clinical EAE signs. After that, on the day 20 after induction of EAE (peak of the disease), mice were euthanized under deepening anesthesia (i.p.), and splenocytes and peritoneal cells were removed. Cells were cultured in triplicates at 37°C and 5% CO_2, in the presence or absence of $MOG_{35–55}$ peptide (10 µg/mL) (Sigma Chemical Co.), and supernatants were collected, and oxygen radicals (NO and H_2O_2) and cytokines (IFN-γ, IL-17 and TNF-α) were measured, respectively, in peritoneal cells and splenocytes.

Clinical assessment

Mice were weighed and observed daily for clinical signs of EAE up to 20 days after immunization (peak of disease). The animals were monitored daily and neurological impairment was quantified on clinical scale of 0 to 5, according to literature (Peron et al., 2010), as follow: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and forelimb paralysis; and 5, moribund state. Results are expressed as the mean ± SD for EAE clinical scores.

Hydrogen peroxide (H_2O_2) measurement

H_2O_2 production by peritoneal cells was assessed according to the adapted method of peroxidase-dependent oxidation of phenol red (Pick & Mizel, 1981). Briefly, after 1h incubation, suspensions of peritoneal cells were mixed with a solution containing 5.5 mM dextrose, 0.5 mM phenol red, 19 U/mL horseradish peroxidase type I RZ 1.0 (Sigma), and 2 mM phorbol myristate acetate (Sigma). The reaction was stopped by adding 10 µL of 1N NaOH solution per well, and the absorbance was measured at 620 nm with a microplate reader (TP Reader NM). Results were expressed as mM of H_2O_2 / 2 x 10^5 peritoneal cells/well.

Nitric oxide (NO) measurement

Peritoneal cells were incubated for 48h and supernatants were removed. NO production was measured according to Griess method (Green et al., 1982), which assesses accumulation of nitrite. Briefly, supernatants were mixed with an equal volume of Griess reagent, which was prepared by mixing one part of 0.1% (w/v) N-(1-naphthyl) ethylenediamine with one part of 1% (w/v) sulfanilamide in 5% phosphoric acid. After 20 min, absorbance was measured at 540 nm using a microplate reader (TP Reader NM). The nitrite concentration was calculated using sodium nitrite as a standard.

Determination of cytokines production

The concentrations of TNF-α, IL-17, and IFN-γ in supernatants splenocytes after 24h of incubation were measured by ELISA method, according to the manufacturer's recommendation (PeProtech Inc, New Jersey). The following solution was applied to visualize binding: 100 µL of ABTS (3-ethylbenzthiazoline-6-sulphonate) (Sigma Chemical Co., Saint Louis, EUA) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.01% H_2O_2. The optical density was measured at 405 nm with spectrophotometer (TP Reader NM microplate reader). The levels of sensitivity for TNF-α, IL-17, and IFN-γ kits were 16 pg/mL (according to the manufacturer's information).

Histological assessment of CNS tissue

For histopathological analysis, CNS samples of brain, cerebellum and spinal cord were dissected from the mice and fixed in 4% formalin. Following dissection, CNS samples were dehydrated in step-up concentrations of ethanol (70%, 90% and 100%) in baths of 1 h each, and 3 baths were performed in absolute alcohol. The samples were then bleached in 3 baths of xylol (1 h each) and finally underwent impregnation in paraffin in an autoclave at 58°C and inclusion in paraffin at room temperature. The blocks were then sectioned into 5 µm of thickness, stained with hematoxylin and eosin, to assess inflammatory damages, as previously described (Corrêa et al., 2010). Histopathological examination was performed out by means of a double-blind study by two different pathologists. Photomicrographs were captured using the Axiostar plus (Camera Sony DCR-PC 100 e Software Axiovision release® version 4.8). After observation, they were selected for digital capture significant areas of the morphology of each body, taking care to shoot the same anatomical locations in all samples.

Statistical analysis

Statistical tests were performed with Graphpad Prism software. Significant differences were determined by a one-way analysis of variance (ANOVA) and by applying Student's t-test for multiple comparisons with the level of significance set at $P < 0.05$.

Results and discussion

LicoA is a chalcone found in licorice with high therapeutic potential, possessing anti-inflammatory, antimicrobial and antitumor properties (X. Chu et al., 2012). In the previous work, we have reported that LicoA displays *in vitro* immunomodulatory activity in EAE cells (Fontes et al., 2014).

Before the *in vivo* study, the concentration of LicoA in brain and serum was determined in order to investigate if its oral administration reaches the target organ. The HPLC method was successfully employed to determine LicoA in mice serum and tissue samples (Figure 2). The analysis of LicoA showed a mean of 0.27 µg/mL of this compound in serum and 0.094 µg/g in brain tissue of mice, one hour after gavage administration.

Analyzing the penetration between tissue/plasma, our results showed that about 36.2% of LicoA present in serum reached the mice brain, suggesting a good distribution in this tissue (Table 1). In this regard, Nadelmann et al. (Nadelmann et al., 1997) were the only ones that reported a method to quantify LicoA in biological fluids (blood and urine of rats) by HPLC-UV. To our knowledge, this study is the first to quantify this molecule in brain tissue using an *in vivo* model. These preliminary findings support the use of this molecule to treat neurological diseases, such as EAE.

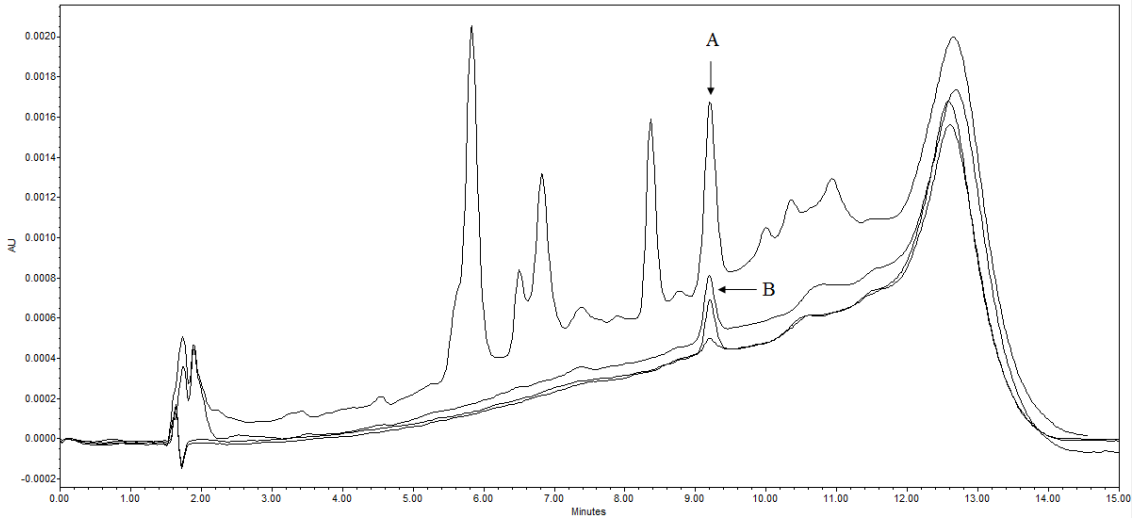

Figure 2. Typical Chromatograms of LicoA in mouse serum (A) and brain tissue (B) compared to standards (0.1625 µg/mL and 0.0325 µg/mL).

Table 1. LicoA determination in serum and brain tissue of mice.

Animal	1	2	3	4	5	Mean	SD
Serum (µg/ml)	0.41	0.27	0.31	0.17	0.2	0.27	0.09
Tissue (µg/g)	0.08	0.08	0.16	0.05	0.1	0.09	0.04
T/S Ratio	0.19	0.29	0.52	0.31	0.5	0.36	0.14
Ratio (%)	19.24	29.28	52.34	30.71	49.9	36.29	14.26

Then, after quantifying LicoA in brain, we investigated, for the first time, the therapeutic potential of LicoA on EAE-induced C57Bl/6 mice, performing *in vivo* treatments.

To determine the *in vivo* effects of LicoA on the development and severity of EAE, C57BL/6 mice were immunized with MOG_{35-55} in CFA plus *Petussis* toxin. When individual mice developed a clinical score equal to 1, they were randomized and treated with LicoA (15 or 30 mg/kg/day) or vehicle up to 20 days post the initial treatment, and the disease severity of individual mice was monitored and recorded daily, using a clinical score scale (Peron et al., 2010). The immunized mice presented EAE signs, such as weakness/paralysis of tail and limbs, which became apparent around days 9-10 after immunization. Mice developed clinical signs with 100% incidence in the no-treated EAE group (Figure 3). When LicoA at 30 mg/Kg/day was administered for consecutive days, severity of EAE was shown to be lower than EAE group from the beginning of treatment and the reduction was significantly reduced in days 13 to 20 (Figure 3). In addition, after LicoA *in vivo* treatment with 30 mg/kg/day, there was a noticeable delay in the disease onset, where mice began to present clinical scores only from day 13, unlike the EAE group, which began in the ninth day after induction, suggesting strong effect suppression (Figure 3). EAE mice treated with 15 mg/kg/day of LicoA also exhibited the first clinical symptoms on day 09 (Figure 3). After, clinical scores were increasing until the disease peak and they were not significantly reduced in comparison with the EAE group. In addition, increasing the dose resulted in an additional effect, since the most effective dose of LicoA (30mg/kg/day; p.o.) was able to suppress acute EAE for more five consecutive days. Also, even after disease onset, LicoA-treated EAE mice at 30 mg/kg/day displayed mild symptoms and some of them were asymptomatic.

Clearly, treatment with LicoA significantly reduced the clinical scores and the mean clinical scores in the LicoA-treated mice were lower than that in the vehicle-treated EAE mice throughout the observation period (Figure 3). On the day 20 after induction of EAE, control animals began to reduce their clinical score, and because of that, they were sacrificed.

The clinical course of MS is characterized by acute relapses during which scattered inflammatory demyelinating lesions within the CNS produce varying combinations of neurological dysfunction (Lassmann, 2011; Mix et al., 2010). Treatment that has the potential to reduce the neurological sequelae of acute relapses will be of benefit to MS patients, as it will improve the natural outcome and decrease neurological disability over time (Chen et al., 2010). In the present study, we showed that in the EAE model, LicoA, at its highest dose, reduced disease severity when given after the onset of clinical signs.

Also, the effects of the *in vivo* treatment with LicoA on the body weight are shown in Figure 4. The mean of body weight in vehicle-treated EAE mice (EAE group) was significantly lower than that observed in the negative control group after 14 days post MOG_{35-55} immunization ($P<0.05$). Although in the LicoA-treated group (15 mg/kg/day) the mean of body weight was higher than that observed in EAE group until 17th day after treatment, but the difference was not statistically significant. Similar pattern of the loss of body weight has been observed for LicoA-treated group (30 mg/kg/day) as compared with EAE group. However, interestingly, LicoA administration (30 mg/kg/day) reversed the trend, and the mean of body weight in this group was significantly lower (15 to 18 days after induction) than that observed in vehicle-treated EAE mice (EAE group).

Next, we examined H_2O_2 and NO production after *in vivo* treatment with LicoA. According to our results, LicoA-treated mice also showed lower levels of oxygen radicals (Figure 5). It was observed that, after *in vivo* treatment, H_2O_2 and NO production was significant inhibited by LicoA (30 mg/kg/day) in comparison with EAE group ($p<0.01$) (Figure 5), which displayed high levels of oxygen radicals in comparison with the negative control group to NO and similar to negative group in H_2O_2 (Figure 5). In contrast, significantly higher levels of oxygen radicals were detected in EAE-group, as compared with that in the negative control group ($p<0.001$) (Figure 5).

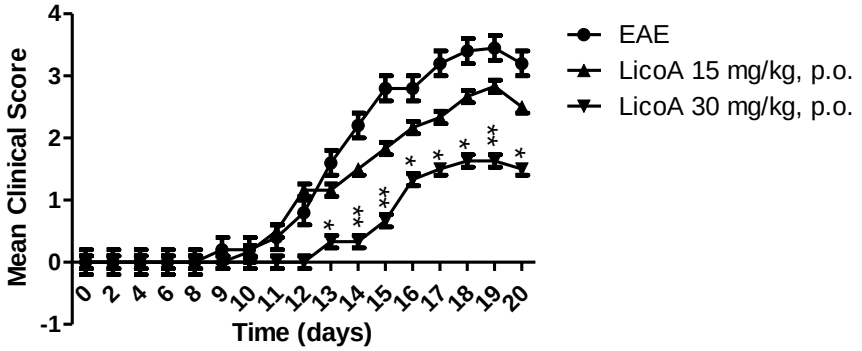

Figure 3. Effects of LicoA on clinical score of treated EAE-induced mice. Results were expressed as mean ± standard deviation (SD) and were considered statistically significant results with $p< 0.05$, $p < 0.05$ (*), $p < 0.01$ (**).

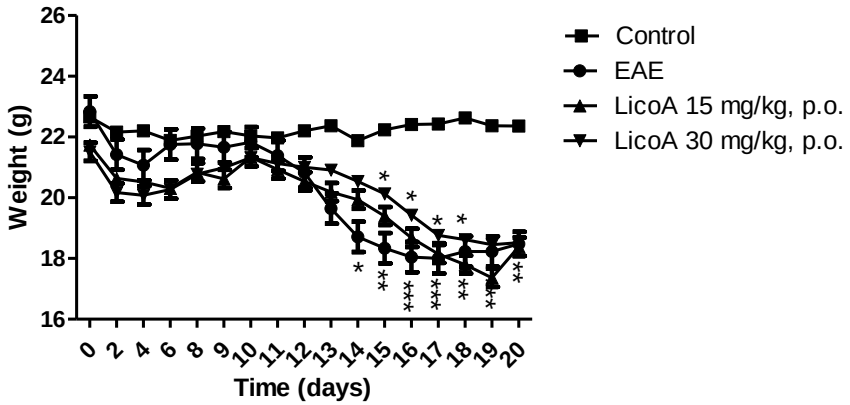

Figure 4. Effects of LicoA on weight loss of treated EAE-induced mice. Results were expressed as mean ± standard deviation (SD) and were considered statistically significant results with $p< 0.05$. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

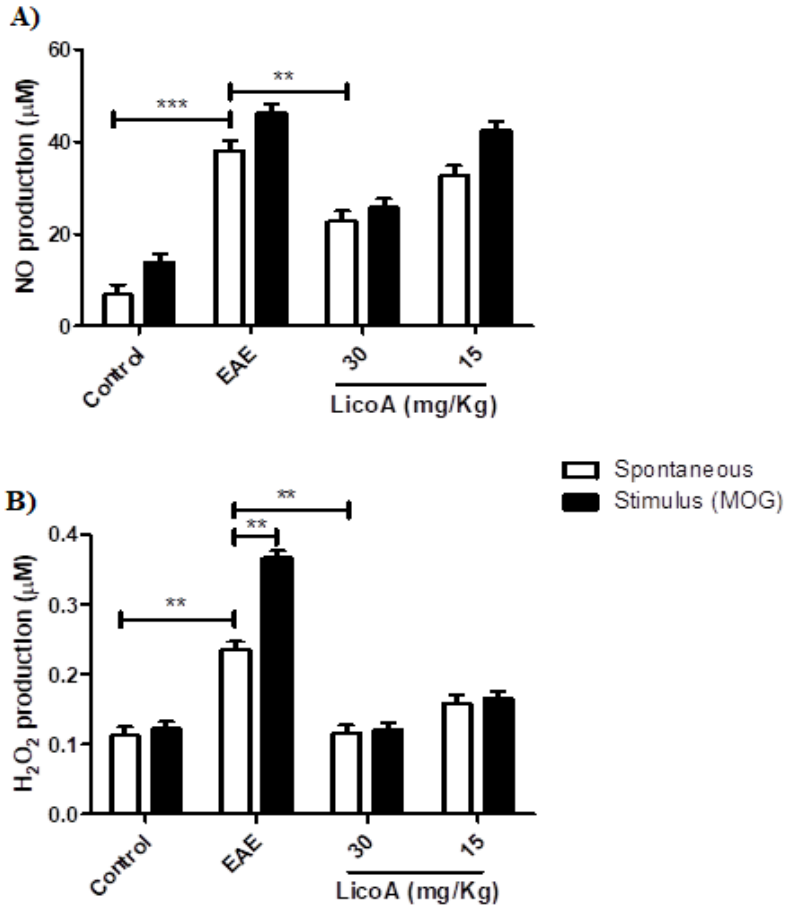

Figure 5. Effects of LicoA on ROS production by peritoneal cells from treated EAE-induced spontaneously and after MOG stimulus. A) NO production. B) H_2O_2 production. Results were expressed as mean ± standard deviation (SD) and were considered statistically significant results with $p< 0.05$. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Taking into consideration $MOG_{35–55}$ stimulus, significantly higher levels of H_2O_2 production were detected in EAE-group (Figure 5B), while H_2O_2 production was inhibited by LicoA (at both tested doses), which suggested that both treatments inhibited the stimulus production. On the other hand, NO production (Figure 5A) was not significantly inhibited by any treatment.

Excessive exposure to reactive oxygen species (ROS) causes oxidative stress. Additionally, excessive ROS production inflicts damage on essential cellular macromolecules including lipids, proteins, and DNA; this damage results in several human diseases, such as inflammation, cancer, atherosclerosis, rheumatoid arthritis, and neurodegenerative diseases (Reuter et al., 2010).

According to literature, NO and H_2O_2 are responsible for direct injury to the myelin sheath in both MS and EAE (Fontes et al., 2014; Leiper & Nandi, 2011; Zha et al., 2022). NO is produced in

large amounts by macrophages and other immune system cells, presenting cytotoxic and cytostatic properties (Leiper & Nandi, 2011). It was reported that LicoA significantly inhibited the expression of the *iNOS* and NO production induced by LPS in RAW264.7 cell culture supernatant (Kwon et al., 2008). Considering that *iNOS* is the essential enzyme for the biosynthesis of NO (Anavi & Tirosh, 2020; Leiper & Nandi, 2011), the lower levels of NO production in groups treated with LicoA could be related to its *iNOS* expression inhibition. In this regard, Huang et al. (Huang et al., 2017) showed that *in vitro* treatment with LicoA inhibited mRNA expression of *iNOS* in a dose-dependent manner and this result demonstrate that the neuroprotective effects of LicoA are associated with microglia and anti-inflammatory effects in Parkinson's disease models.

Also, active oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical (OH·), are formed through a one-electron reduction process of molecular oxygen (O_2). They are generated by a process known as redox cycling, and are catalyzed by transition metals, such as Fe_2^+ and C_u^+ to cause DNA damage, thiol oxidation and lipid peroxidation that can lead to cell death (Quintanilha, 1988). Therefore, ROS clearance and oxidative stress inhibition may play essential roles in preventing numerous diseases, like EAE.

In this regard, LicoA is a compound that possesses radical-scavenging and antioxidant effects (Haraguchi et al., 1998) and, considering that as it reduced NO and H_2O_2 production, even after stimulation, those effects may be related to the antioxidant properties of LicoA. This was also showed by Huang et al. (Huang et al., 2017), were BV-2 microglial cells treated with LPS for 24h displayed a marked increase in NO and PGE2 production and NO and PGE2 production was dose-dependently inhibited by LicoA pretreatment in the Parkinson's disease models.

Analyzing the results of cytokine productions, higher levels of TNF-α, IFN-γ and IL-17 were observed in the EAE group when compared with the CN group ($p<0.001$) (Figure 6A–6C). Also, the levels of IFN-γ and TNF-α (Figure 6A–6B) were significantly reduced in relation to the EAE group ($P<0.01$). Also, after *in vivo* treatment with LicoA (15 and 30 mg/Kg), IL-17 production was significantly inhibited ($p<0.05$ and $p<0.01$, respectively), demonstrating that LicoA exercised strong suppressive activity in this cytokine (Figure 6C).

Regarding $MOG_{35–55}$ stimulation, LicoA was not able to present different productions among the spontaneous groups, compared with those stimulates with $MOG_{35–55}$ to TNF-α and IL-17 production (Figure 6B and 6C). Still under consideration to $MOG_{35–55}$ stimulus, significantly higher levels of IFN-γ production were detected in EAE-group and LicoA treated with 15mg/Kg/day. Regarding to LicoA treatment with 30 mg/mg/day during EAE induction the results showed that the treatment inhibited the stimulus production (Figure 6A).

Cytokines and chemokines play important roles in the establishment and maintenance of autoimmune disorders, such as in MS and EAE (Lassmann, 2011; Mix et al., 2010; Papiri et al., 2023). In EAE, T cells secrete pro-inflammatory cytokines and chemokines, resulting to demyelination through the activation of macrophages and microglia (Poppell et al., 2023; Rodgers & Miller, 2012). When microglia and macrophages are activated, they release some cytotoxic mediators that may provoke tissue injury, including IFN-γ, TNF-α, NO, and ROS (Fletcher et al., 2010; Rodgers & Miller, 2012).

IFN-γ is the cytokine involved in both macrophage activation and T cell differentiation in naïve $CD4^+$ Th1 cells, as well as it regulates the functions of T lymphocytes, playing an important role in autoimmune diseases, such as EAE and MS (Lee et al., 2012; Rodgers & Miller, 2012). Likewise, TNF-α is a cytokine present in Th17 and Th1 cells that plays an essential role in both MS and EAE (Lassmann, 2011; Rodgers & Miller, 2012). It has been reported that LicoA is capable of inhibiting the generation of reactive oxygen species (ROS), as well as the secretion of some inflammatory cytokines, such as TNF-α, through the inhibition of the activation of NF-κB (Kwon et al., 2008).

Also, LicoA has demonstrated several biological activities, exhibiting anti-inflammatory properties (Chen et al., 2017; Hu & Liu, 2016; Kwon et al., 2008; M. Liu et al., 2024; Shen et al., 2024; Su et al., 2018), as well as anticancer and antioxidant effects (Chen et al., 2017; Su et al., 2018).

According to literature, LicoA significantly inhibited LPS-induced NF-κB activation. LicoA has protective effects against LPS-induced acute kidney injury and LicoA exhibits its anti-inflammatory effects through inhibiting LPS-induced NF-κB activation (Hu & Liu, 2016). The nuclear factor NF-κB is a central regulator of inflammatory processes, encoding the genes of some proinflammatory cytokines, such as TNF-α, which is an important mediator in the process of neuroinflammation, as well as responsible for some of the harmful effects of brain injuries and neurological diseases (Guo et al., 2014).

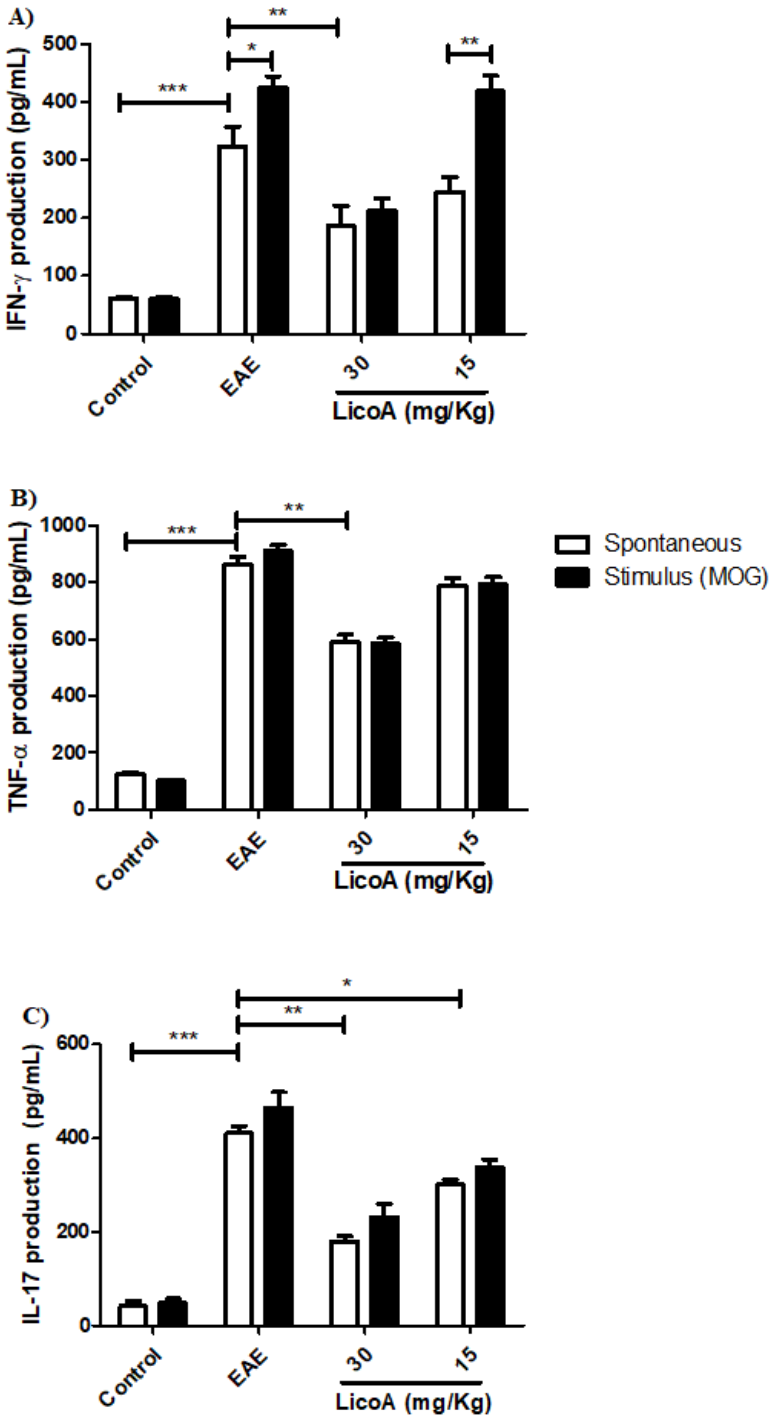

Figure 6. Effects of LicoA on cytokine production by splenocytes from treated EAE-induced mice spontaneously and after MOG stimulus. A) IFN-γ production. B) TNF-αproduction. C) IL-17 production. Results were expressed as mean ± standard deviation (SD) and were considered statistically significant results with $p< 0.05$. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Corroborating with this, (Huang et al., 2017) showed that LicoA exerted potent neuroprotection on dopaminergic neurons against LPS-induced neurotoxicity in Parkinson's disease models *in vivo* and *in vitro*. The underlying proposed mechanism is that LicoA inhibits LPS-induced microglial activation via downregulation the activation of ERK1/2 and NF-κB p65 pathways and, after, reducing the production of NO, TNF-α and IL-6.

Here, we report for the first time that LicoA inhibits IL-17 production from MOG_{35-55} immunized mice. Recent studies have emphasized the importance of pathogenic self-reactive Th17 cells in EAE (Chen & Shannon, 2013; Kong et al., 2014). According to literature, in addition to proinflammatory cytokine release (IL-17) and neutrophil recruitment, Th17 cells also induce neuronal cell death directly, being one of the main cell types involved in autoimmune diseases, such as

MS and EAE (Siffrin et al., 2010; Wang et al., 2013). Similarly, IL-17, produced by Th17 cells, has been strongly linked to the pathogenesis of MS, where it exerts direct cytotoxic activity (Komiyama et al., 2006; Siffrin et al., 2010). In addition, it has been shown that IL-17 levels were increased at the sites of lesions of MS patients and that deficiency or neutralization of IL-17 delays the onset of EAE and reduces the severity of the disease (Komiyama et al., 2006).

Th17 differentiation is driven by cytokines TGF-β, IL-6, and IL-23, resulting in SMAD and Stat3 phosphorylation and subsequent RORγt transcription (O'Shea et al., 2009). IL-17 induces chemokine production by cells, attracting neutrophils to the sites of inflammation (Karczewski et al., 2016).

The first evidence of the importance of Stat3 in regulating IL-17 production came from the demonstration that the IL-17A and IL-17F locus has multiple putative Stat binding sites. Using chromatin immunoprecipitation assays, it was determined that Stat3 directly binds to this promoter (Chen et al., 2006). Furthermore, *in vitro* Th17 differentiation is greatly impaired in Stat3-deficient T cells (Yang et al., 2007). Thus, Stat3 is a direct regulator of IL-17 (O'Shea et al., 2009).

As reported, LicoA significantly inhibited the phosphorylation and nuclear localization of Stat3 (Funakoshi-Tago et al., 2008), which is essential for TEL-Jak2-induced cell transformation. These data suggest that LicoA is a specific inhibitor for Stat3 and, therefore, it could be employed for the treatment of various diseases caused by disorders of the Jak/Stat pathway (Funakoshi-Tago et al., 2008). Therefore, it can be suggested that LicoA reduced severity of EAE mainly through IL-17 inhibition in addition to its action on IFN-γ, TNF-αand ROS production.

Collectively, these data indicated that the *in vivo* treatment with LicoA suppresses the development and severity of EAE in mice, as well as LicoA ameliorated clinical scores when given orally after symptoms development. To confirm whether LicoA treatment provides therapeutic effects on EAE-mice, we examined the pathological changes in CNS samples of mice by histopathology (Figure 7).

Evaluation of the white matter of the spinal cord, brain, and cerebellum in the control group stained with hematoxylin–eosin showed typical neuropil with a large network of glial cell prolongations and cell prolongations originated in neuron bodies in the cortical areas (Figure 7A,E,I). On the other hand, a massive inflammatory infiltrate within the CNS tissues of vehicle-treated mice (EAE groups) was evident (Figure 7B,F,J). Samples of EAE groups stained with HE presented an intense perivascular inflammatory infiltrate in several cortical and white matter areas, composed predominantly of mononuclear cells with round-shaped, intensely basophilic nuclei and scarce cytoplasm. Associated with this infiltrate there were accumulations of cells with well stained fusiform, flat nuclei associated with microgliosis. Especially in the spinal cord white matter, the neuropil presented more space between prolongations and more glial cells when compared with the control group (Figure 7B,F,J).

However, in LicoA treated group with 15 mg/Kg/day (Figure 7C,G,L) it is possible to note capillaries surrounded by mononuclear inflammatory infiltrate, predominantly lymphocytic. In these samples, the presence of perivascular inflammation in the subarachnoid was highlighted (Figure 7C). In areas of gray matter, areas of greater glial cellularity were observed in the brain cortex, while in the white matter, fewer myelin fibers were found in areas of fibrillar disarray compared to samples from the negative control group (Figure 7G).

In the spinal cord of the animals in the LicoA treated group (30 mg/kg/day), areas of gray matter compatible with normality were observed. In the white matter, the typically myelinated areas were also compatible with normality in both quantity and structuring as well as glial cellularity. However, it is noteworthy that in some few points a dispersed mononuclear inflammatory infiltrate was observed (Figure 7D). A similar result was observed in the white matter of the encephalon (Figure 7H), where discrete accumulations of mononuclear leukocytes were visualized. However, these findings were clearly less intense than in the EAE group, as well as neuropil with more sparse prolongations. Also, it is observed mild glial cells associated with cells whose morphology is suggestive of microglia and astrocytes (Figure 7C,G,H,L). Interestingly, evaluation of the samples from LicoA 30 groups (Figure 7D,H,M) showed typical neuropil with a large number of myelinated fibers with sparse areas of few focal inflammation, or even absence of inflammatory infiltrated (Figure 7D,G,M). Therefore, in addition to the improvement of clinical scores, histological examination indicated that LicoA treatment (mainly at the 30mg/Kg/day) reduced the numbers of inflammatory infiltrates and mitigated the EAE-related demyelination in the CNS of mice.

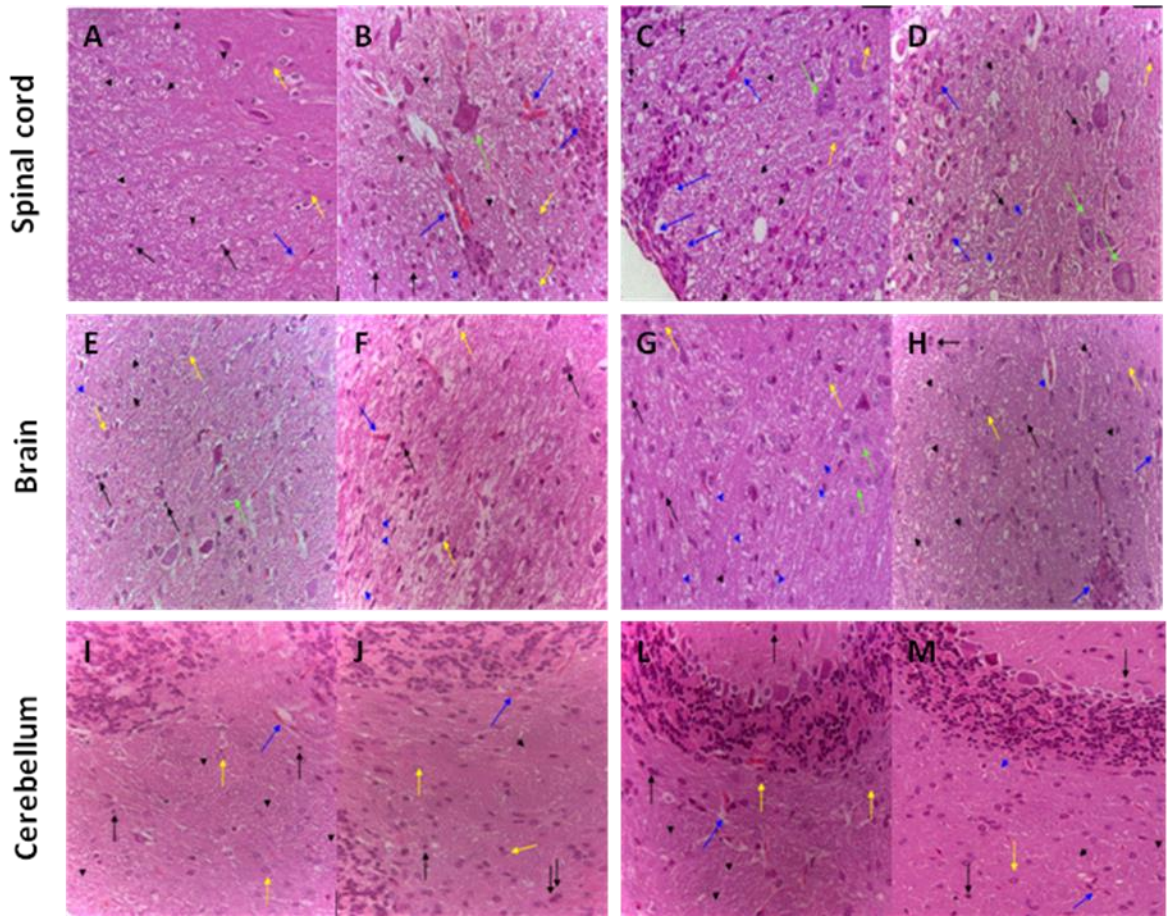

Figure 7. Histological patterns of inflammatory infiltrate into the CNS. CNS samples (spinal cord, brain and cerebellum) were collected from normal (control), EAE mice treated with vehicle (EAE), 15 mg/kg or 30 mg/kg of LicoA. The sections were stained with hematoxylin and eosin to examine the inflammation. Typical images were chosen from each experimental group (original magnification ×400). (A, E, I) control groups; (B, F, J) EAE groups with extensive areas of inflammation; (C, G, L) LicoA 15 mg/kg, mild inflammatory damages; (D, H, M) LicoA 30 mg/kg sparse areas or absence of inflammation. Black Arrows: oligodendrocytes; Yellow Arrows: astrocytes; Green Arrows: perycarion; Blue Arrows: blood vessels; Black Arrow Heads myelin fibers - longitudinal section; Blue Arrow Heads myelin fibers - longitudinal section - cross section.

Conclusion

In summary, the present study provided the first clinical evidence that LicoA reached the brain after oral administration. Also, the oral treatment with LicoA (mainly at 30 mg/kg/day) significantly ameliorated the inflammatory signs and attenuated the pathology development of EAE in mice. All these findings reveal a noticeable therapeutic effect of LicoA on the clinical course of EAE and open the route to further studies with this compound as prototype for therapeutic of MS.

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