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Effects of lemon balm (*Melissa officinalis*) on behavioral deficits and memory impairment of rats surviving sepsis

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Sepsis has become one of the most frequent causes of mortality in intensive care centres. So far, there is no effective pharmacotherapy that can prevent or improve the neurological consequences and enhance survival. The goal of this study is to investigate the neuroprotective effect of lemon balm (*Melissa officinalis*) on behavioural dysfunctions produced in sepsis-surviving rats. Adult male rats were subjected to caecal ligation and puncture and the control animals were submitted to the sham operation. Lemon balm ethanolic extract or saline, given orally, was administered for one week after surgery procedures. Locomotion, anxiety, depressive behaviour and memory were investigated. In the elevated plus-maze (EPM), the percentage of open arm entries and open arm time was very significant in the animals treated with lemon balm extract, similar to the diazepam response in sham-operated and sepsis-surviving rats. Locomotion in open field tests and the enclosed arm entries in the EPM were not significantly altered by treatments. In the forced swimming (FS) test, the extract was effective at reducing the immobility time as that demonstrated by fluoxetine. In the step-down inhibitory avoidance test, the extract eased the effects on memory of sepsis-surviving animals. Collectively, these results demonstrate that lemon balm ethanolic extract could be used for the prevention of cognitive and mood-related deficits that may be associated with sepsis sequelae.

Key words: Anxiety, depression, locomotion, *Melissa officinalis*, memory.

INTRODUCTION

Brain disorders are currently getting top priority among the science target and the literature has shown the behavioural problems associated with traumatic brain injury. Sepsis is an example of this damage and it is characterized by a systemic inflammatory response and is associated with high mortality in humans (Greenberg et al., 2014). The main reason of death in septic patients could be encephalopathy (Mazeraud et al., 2016; Tauber

et al., 2017). Nowadays, several studies are conducted to evaluate the damage to the brain during sepsis and its behavioural consequences, especially involving cognitive and mood impairments present in sepsis survivors (Comim et al., 2016; Leite et al., 2013; Mazeraud et al., 2016; Tauber et al., 2017). The polymicrobial sepsis induced in rats by caecal ligation and perforation (CLP) experimental models are important because they reproduce human sepsis consequences (Leite et al., 2013), which is clinically relevant for elucidating new therapy alternatives for this health problem (Hutchins et al., 2014).

Traditional medicine was used on people before the great development of modern medicine, based on multiple alternatives of natural products to treat several physiological dysfunctions. Research into the historical literature has demonstrated that some of the natural products and/or their pharmacologically active principles have a broad spectrum of biological activities, including central nervous system (CNS) properties (Andrade et al., 2016; Bu et al., 2016).

Piper nigrum L. (Piperaceae) used in traditional medicine of many countries, for example, has different properties, such as antiinflammatory, antioxidant, antidepressant and cognitive effects. The methanolic extract of their fruits in beta-amyloid rat model of Alzheimer's disease significantly exhibited anxiolytic- and antidepressant-like effects and also antioxidant potential (Hritcu et al., 2015). Acute treatment of rats with *Lindera obtusiloba* extracts, commonly used as an alternative medicine in Korea, significantly reduced immobility time and increased swimming time, suggesting that the antidepressant-like effects of this extracts were likely mediated via the glucocorticoid receptor (Lim et al., 2016). In addition, other neuroprotective efficacy could be observed in *in vivo* and *in vitro* experiments (Jalsrai et al., 2016).

Melissa officinalis is a medicinal plant from the Lamiaceae family that has been used as a folk medicine to threat central nervous disorders (Anheyer et al., 2017; Kennedy et al., 2002). This plant has neuroprotective activity at CNS because it displays potent antioxidant activity (Soodi et al., 2017). From the phytochemical point of view, the main chemical principles of this species of herbal medicine are flavonoids, alkaloids, phenolic acids and tannins (Noguchi-Shinohara et al., 2015; Shakeri et al., 2016). These compounds may contribute to the homeostasis of the CNS, especially because they can protect against neurodegeneration and/or behavioural disorders that can be correlated to dementia or traumatic brain injury. Notably, it has also been reported as lowering excitability, cognitive dysfunctions, stress and anxiety levels in rodent models and humans (Anheyer et al., 2017; Lin et al., 2015; Ross, 2015; Shakeri et al., 2016). Previous results from our lab showed that the oral intake of lemon balm EE for about 10 days has anxiolytic and antidepressant-like properties in a manner gender (male vs. female) dependent, particularly with regard to the effective dose for each exposure length (Taiwo et al., 2012).

Despite great availability of information regarding the neuroprotective effects of plant extracts, the options for sepsis treatment is scarce. In this context, the goal of this work is to verify the possible neuroprotective action of lemon balm extract on behavioural and cognitive deficits due to sepsis sequela in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (n= 60), 3 months old, 350 g, from the animal facility of Sena Aires Faculty (Valparaíso de Goiás-GO, Brazil), were housed 5 to a cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 am) at room temperature (23 ± 1°C). The animals were allowed to adapt to the laboratory conditions for at least 1 week before the behavioural assessment. All procedures used in the present study complied with the guidelines on animal care of the UNB Ethics Committee on the Use of Animals, which follows the “Principles of laboratory animal care”. Subjects were transferred in their cages to the experimental room on the day of being tested, where they were left unbothered to habituate for at least 1 h before the behavioural tests.

Preparation of the plant extract

Dried leaves of *M. officinalis* were kindly supplied by Centroflora Group (Botucatu, Brazil), who also provided a certification of the plant’s identity and quality. Powdered plant material (1900 g) was extracted by maceration at room temperature (24±3°C) using ethanol as solvent. Following removal of the solvent, under vacuum conditions and below 40°C using a Heildolph system (Heildolph ® Instruments, Germany) composed by a rotavapor coupled to a vacuum pump D-91126, and chiller a MX07R-20HD2E. After solvent elimination, the crude ethanolic extract (EE) that was obtained (13% yield) was then stored at -18°C.

Drugs and pharmacological procedures

Lemon balm EE (100 mg/kg) was dissolved in a solution containing 150 µL of Tween 80, 150 µL of ethanol and 150 µL of Dimethylsulfoxide (DMSO). All these substances (except EE) were obtained from Sigma Aldrich, Brazil. Each resulting preparation was then suspended in 0.9% physiological saline. An equivalent

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preparation of Tween/alcohol/DMSO/saline (this mixture was used to avoid fluid overload, swelling, acidosis and high blood sodium, and to produce the best dilution of the drugs. Saline was used to dilute other medications to be given by injection) was used as vehicle control. These solutions were prepared 24 h prior to being administered via oral gavage, in a volume of 1 mL/kg, having been stored at 4°C. Diazepam (1 mg/kg; DZP; Roche, Brazil) and fluoxetine (10 mg/kg; FXT; Bluepharma, Brazil) were suspended in distilled water containing 2% Tween 80 and physiological saline (DMSO is not necessary in this solution, only for lemon balm EE due to the product consistency), respectively. Both drugs were prepared on the same day as being administered by gavage, in a volume of 1 mL/kg. Lemon balm EE dose selection was based on previous study published elsewhere (Kennedy et al., 2002). The doses of the other substances varied according to the pharmaceutical efficacy of each of them, in accordance with clinical practice.

Caecal ligation and perforation surgery

Rats were weighed and anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. A 2-cm midline laparotomy was performed to allow exposure of the caecum to the adjoining intestine. The caecum was tightly ligated 5 times with a 4.0-silk suture (Bioline, Brazil) at its base, below the ileocaecal valve, and it was perforated five times through-and-through with a 14-gauge needle (BD, Brazil). The caecum was then gently squeezed to extrude a small amount of faeces from the perforation site, returned to the peritoneal cavity, and the laparotomy was closed with 4.0 silk sutures. The sham-operated rats were submitted to all surgical procedures previously described but the caecum was not perforated. To reproduce the protocol used in surgery procedures on humans, all animals received saline immediately after surgery procedures (50 mL/kg subcutaneous). A mixture of ceftriaxone (30 mg/kg) + clindamycin (25 mg/kg) was administered by intraperitoneal route (i.p.) every 12 h for 3 days. They were kept in groups of 5 animals per cage with free access to food and water, according to the procedure performed elsewhere (Leite et al., 2013).

Treatment protocols

The animals were classified into 2 groups: the sham-operated group and the sepsis group. Each one was divided into 3 subgroups to receive by oral route the control solution (saline); diazepam (1 mg/kg) or fluoxetine (10 mg/kg), as positive controls; or lemon balm EE (100 mg/kg). Animals received daily treatments for 7 days and they were tested one hour after the seventh administration. On the test day, one hour after treatment, the animals were subjected to the behavioural tests. The doses chosen in these protocols were based on the literature or previous experiments from the laboratory.

Experimental procedures

Open field test

To evaluate locomotor activity, animals were tested in an open field (OF), consisting of a wooden square box (60 x 60 x 35 cm) divided into nine equal squares (20 x 20 cm). Each animal was placed in the centre of the field and left to explore freely for 5 min. The number of quadrants crossed with all four paws was used as a measure of locomotion. After each trial, the apparatus was cleaned with ethanolic solution (10% v/v).

Elevated plus-maze test

Measures of anxiety were obtained in the elevated plus-maze (EPM) test. This apparatus is made of wood, comprised of two opposing open arms (50 x 10 cm) and two opposing closed arms (50 x 10 x 40 cm), interconnected by a common central platform (10 x 10 cm). The entire apparatus was elevated 50 cm above floor level, but to prevent falls, both open arms had a 1 cm high plexiglass edge. The animal was placed on the central platform, facing an enclosed arm, and allowed to explore the maze freely for 5 min. The following parameters were scored: number of open and enclosed arm entries, time spent in the central platform, open and enclosed arms. These were used to calculate the percentages of open arm entries (%OAE) and open arm time (%OAT). Arm entry was defined as all four paws in a specific arm (Pellow et al., 1985). After each trial, the EPM was cleaned with ethanolic solution (10% v/v).

Forced swimming test

The forced swimming (FS) test was used, modified from that described by Porsolt et al. (1977). The animal was placed, for 5 min, in a glass cylinder (30 cm in diameter and 50 cm height) containing 40 cm of water at 23±1°C, which forced the rat to swim or float as its hind limbs were unable to touch the bottom. Time spent immobile was recorded only during the last 3 min. Immobility was scored whenever the animal stopped swimming and floated, making only the small limb movements necessary to keep its head above water level. After 5 min, the animal was removed from the apparatus and dried.

Step-down inhibitory avoidance test

The inhibitory avoidance apparatus (Insight, Brazil) is to evaluate aversive memory. It consisted of an acrylic box (50 × 25 × 25 cm), whose floor was made of parallel stainless-steel bars (1 mm diameter) spaced 1 cm apart. A platform (7 cm wide × 2.5 cm high) was placed on the floor against the left wall. The animals were placed on the platform and their latency in stepping down on the grid with all four paws was measured with an automatic device. The animals were submitted to the inhibitory avoidance task using a protocol similar to that described previously (Lucena et al., 2013). During training sessions, immediately after stepping down on the grid, the animals received a 0.4-mA, 1.0-s (controlled by a monitor attached to the equipment) scrambled foot shock. During test sessions, no foot shock was administered and the step-down latency (maximum 180 s) was used as the measure of retention. The animals were submitted to a single training session.

In order to evaluate memory, test sessions were performed 1.5 h after training. The administration of the extract or control solution was performed by oral route 1 h before the training in the inhibitory avoidance apparatus.

Statistical analysis

Data were expressed as means ± mean standard error (s.e.m.) of 10 animals and were analysed using one-way analysis of variance (ANOVA) followed by Tukey's test. Data on the inhibitory avoidance test are shown as median (interquartile range) of step-down latencies. Comparisons of test session step-down latencies

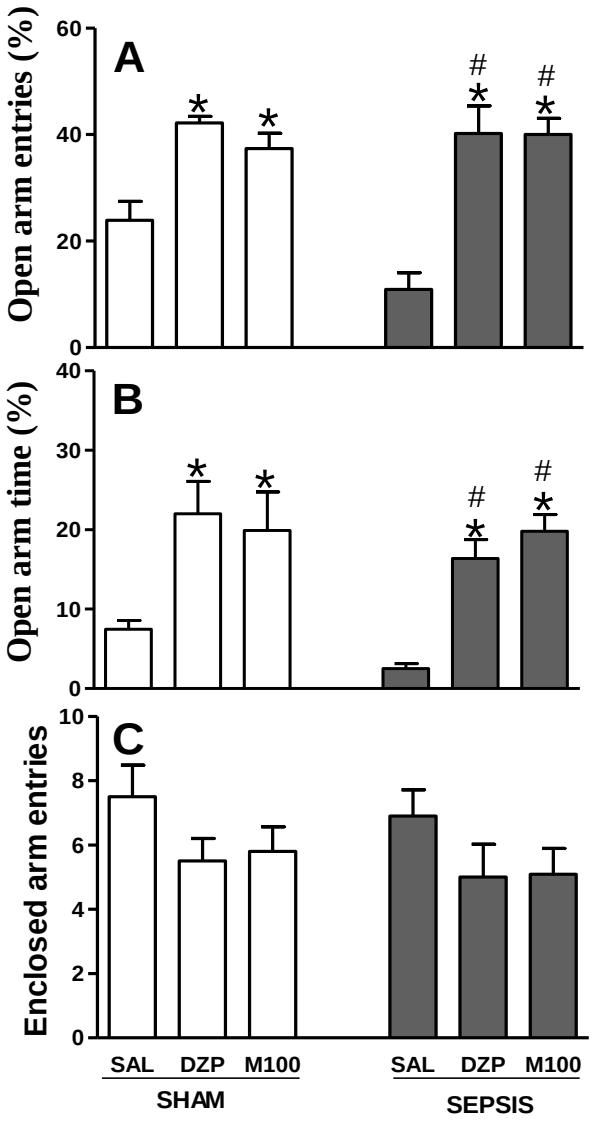

Figure 1. Effects of oral subchronic administration of saline (SAL), diazepam (DZP) and *Melissa officinalis* ethanolic extract (100 mg/kg) on the percentage of open arm entries (A), on the percentage of open time spent (B) and on the number of enclosed arm entries (C) in the elevated plus-maze test on sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group. *$p \leq 0.05$ as compared to the saline treatment of the SHAM group; #$p \leq 0.05$ as compared to the SEPSIS group (ANOVA, Tukey's test).

between groups were performed with the Kruskal–Wallis test followed by the Mann-Whitney test. Data were analysed by Graph Pad Instat® (GraphPad software, San Diego, CA). P-values lower than 0.05 ($P<0.05$) were considered statistically significant.

RESULTS

In the EPM, sham-operated rats that had been exposed to lemon balm EE showed enhanced percentage of open arm entries (Figure 1A): [$F_{(5,59)}$ = 5.00, p<0.05] and percentage of open arm time (Figure 1B): [$F_{(5.59)}$ = 4.20, p<0.05], similar to the animals treated with DZP: % open arm entries (Figure 1A): [$F_{(5.59)}$ =5.06, p<0.05] and % open arm time (Figure 1B): [$F_{(5,59)}$ = 4.91, p<0.05]. In the sepsis-surviving rats, the extract also enhanced the percentage of open arm entries (Figure 1A): [$F_{(5.59)}$ = 5.91, p<0.05] and the percentage of open arm time (Figure 1B): [$F_{(5.59)}$ = 5.07, p<0.05], similar to the animals treated with DZP: % open arm entries (Figure 1A): [$F_{(5.59)}$ =5.96, p<0.05] and % open arm time (Figure 1B): [$F_{(5.59)}$ = 5.00, p<0.05]. The frequency of enclosed arm entries (Figure 2C) in the EPM as well as the number of quadrants crossed in the open field test (Figure 2) were not affected by any treatment and remained constant between groups.

As shown in Figure 3B, FXT treatment was able to reduce the immobility time in sham-operated ([$F_{(5.59)}$ = 3.96, p<0.05]) and sepsis-surviving rats ([$F_{(5.59)}$ = 3.21, p<0.05]) as compared to the group that received the control solution in each group. Lemon balm EE in sepsis-surviving rats modified the immobility time only in the sepsis groups ([$F_{(5.59)}$ = 5.37, p<0.05]).

As shown in Figure 4, the Kruskal-Wallis test followed by the Mann-Whitney test revealed that the lemon balm EE administration was able to improve the memory-facilitating effect produced by sepsis-surviving rats in the model of the step-down inhibitory avoidance test when measuring short- ([H(4, N=31) = 27.10, p<0.0001]) and long-term memory -([H(4, N=31) = 24.80, p<0.0001]).

DISCUSSION

Lemon balm EE was effective in reducing anxiety, depression and cognitive impairment levels produced by sepsis in rodents, showing a property independent of any sedative effect as demonstrated by no interference in locomotion in the open field test and frequency of enclosed arm entries in the EPM, as demonstrated by Figures 1 to 4. Most of the time, the phytomedicine showed a similar effect with the positive controls DZP and FXT, suggesting that the interference in GABA and 5-HT receptors by this extract may be a new alternative for treating neurological dysfunctions produced by sepsis.

Anxiety and depression are the most prevalent mental disorders arising among child, adolescent and adult patients. Both psychiatric illnesses demonstrate marked overlap and co-occurrence (Freeman et al., 2002). Despite this, the prevalence of anxiety is 15 to 25% and the prevalence of depression appears to be higher than 50% (Brown et al., 2001; Lewinsohn et al., 1997). These two disorders may share the same or different vulnerability factors even though anxiety disorders may represent causal risk factors for the development of depression (Goes, 2015; Starr et al., 2014).

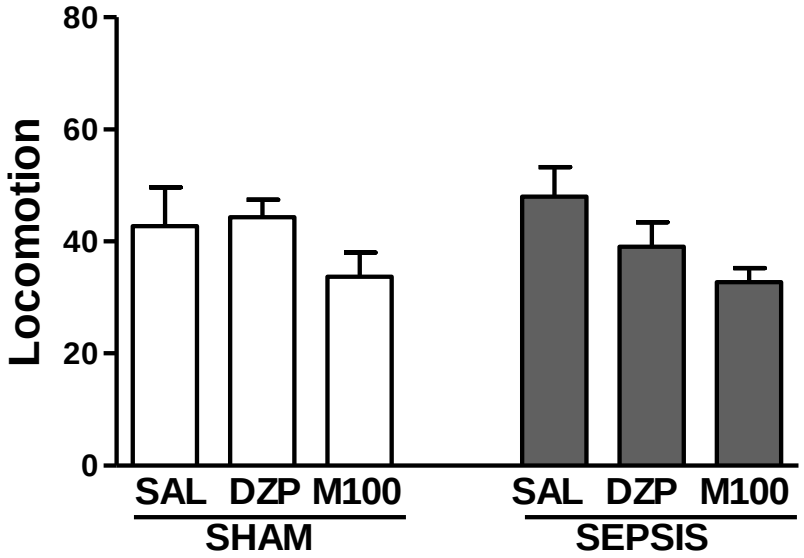

Figure 2. Effects of oral subchronic administration of saline (SAL), diazepam (DZP) and *M. officinalis* ethanolic extract (100 mg/kg) on the number of quadrants crossed in open field test in sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group.

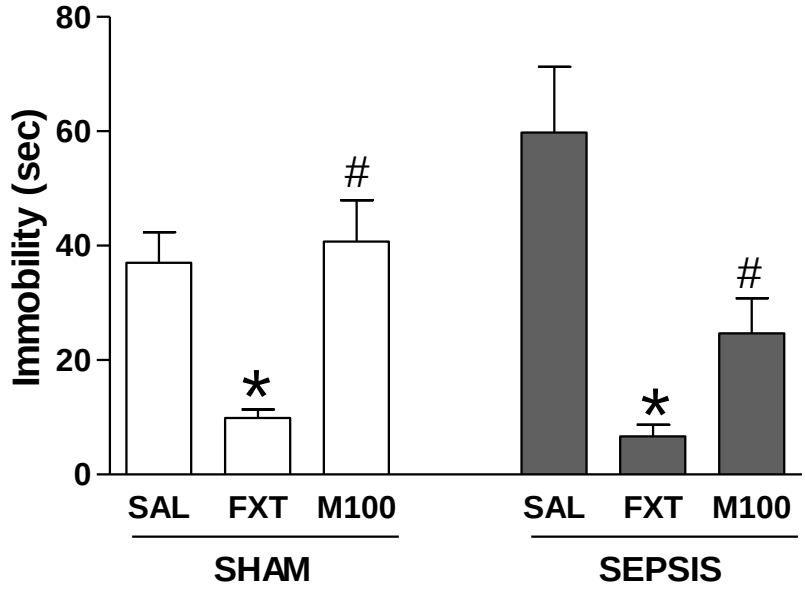

Figure 3. Effects of oral subchronic administration of saline (SAL), fluoxetine (FXT) and *Melissa officinalis* ethanolic extract (100 mg/kg) on the immobile time (in seconds) evaluated in the forced swimming test in sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group. *$p \leq 0.05$ as compared to the saline treatment of the SHAM group; #$p \leq 0.05$ as compared to the FXT group (ANOVA, Tukey's test).

In the context of actual research, it was noted that the sepsis-surviving rats showed suggestive anxiety behaviour that was observed by reduction in the percentage of open arm entries and the time spent in those arms in the EPM, characterizing anxiogenic response. Those rats receiving lemon balm EE maintained a similar response to the

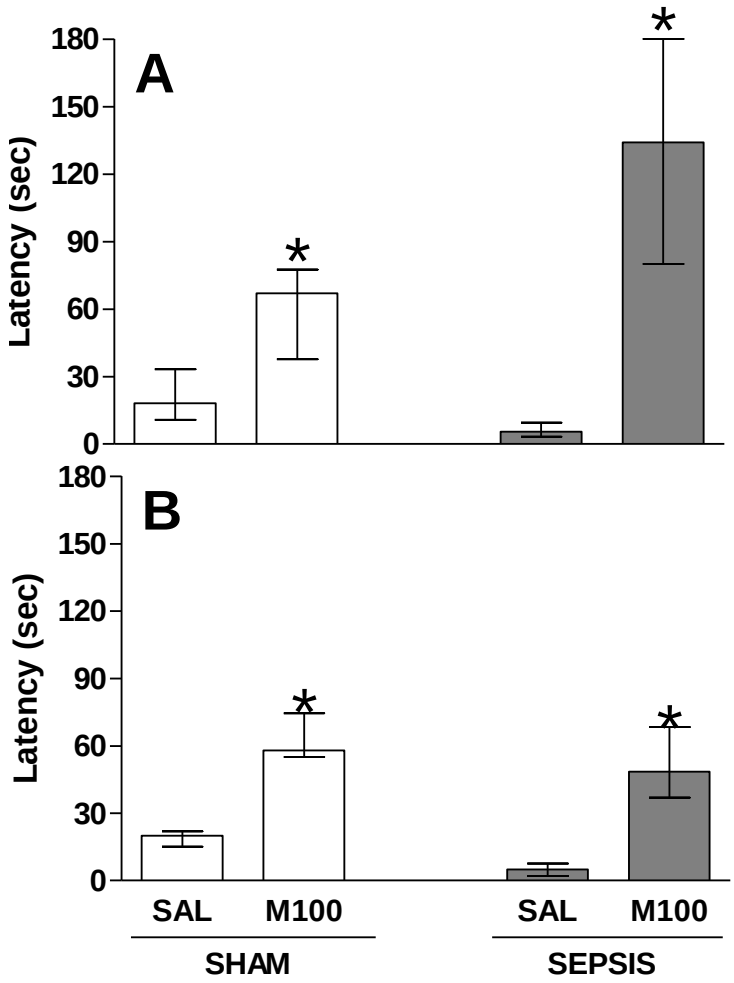

Figure 4. Effects of oral subchronic administration of saline (SAL) and *Melissa officinalis* ethanolic extract (100 mg/kg) on short- (A) and long-term memory (B) in the step-down inhibitory avoidance test in sham-operated and sepsis-surviving rats. Data are shown as the median (interquartile ranges) of 10 animals. *p<0.05 as compared to the saline treatment of the respective group (Kruskall Wallis - Mann Whitney).

sham animals. As regards the assessment of depression, the animals showed increased immobility time in the forced swimming test, which was reduced by FXT and the lemon balm EE. These anxiolytic and antidepressant results were not due to either hypoactivity or hyperactivity displayed by animals, since their locomotion was not changed in the open field test and the entries in the enclosed arms in the EPM.

The observed anxiolytic effect of this natural product is congruent with data obtained elsewhere (Kennedy et al., 2006). The results are supported by the fact that rodents normally exhibit a preference for the enclosed arms and avoidance of the open arms of the EPM. Anxiolytic drugs, such as DZP, modulate EPM behaviour in rodents, causing an increase in the percentage of entries and time spent in the open arms of the maze. Therefore, these measures can serve as an index of anxiety (Horii and Kawaguchi, 2015).

Thus, our results are in accordance with previous data, showing anxiogenic and depressive responses in sepsis-surviving rats. Leite et al. (2013) observed that repeated nicotine administration does not alter the survival rate in rats subjected to caecal ligation and puncture and they provide new evidence that nicotine can improve long-lasting memory impairment and anxiogenic-like responses in sepsis-surviving animals. Tuon et al. (2007), in turn, demonstrated in sepsis-surviving rats, a significant increase in the immobility time as compared to the sham-operated rats. The imipramine was able to reduce this evaluated parameter, with no locomotion impairment. In

the experiments, the FXT, a selective serotonin reuptake inhibitor, showed similar results.

Lemon balm is known for its mild sedative properties (Anheyer et al., 2017), even following a single administration (Soulimani et al., 1991). In our study, such behaviour was not observed. The fact that the lemon balm EE and DZP did not generally alter quadrant crossings and enclosed arm entry in the sham and EPM tests, respectively, suggests that the psychoactive effects observed in the present study may not be due to changes in the animals' locomotor patterns.

Regarding the cognitive aspect, animals that survived the sepsis showed a loss of short- and long-term memory, which was characterized by the decrease in length of time spent in the inhibitory avoidance platform. The administration of lemon balm EE made the animals spend more time on the platform, characteristic behaviour of memory retention.

Several studies have recently shown the importance of phytotherapies for treating behavioural dysfunctions and cognitive problems in humans (Anheyer et al., 2017; Kennedy et al., 2002; Ross, 2015), even though there is little information about the possible pharmacodynamics involved in the pharmacological effects. For *M. officinalis*, it is well documented that its effects could be related to the active components that have been identified as monoterpenoid aldehydes, monoterpene glycosides, flavonoids and polyphenolic constituents (Shakeri et al., 2016; Wightman, 2017).

Substantial progress has been made in our understanding of how some herbal medicines can ameliorate behavioural and cognitive processes in patients with different neurological impairments. As previously mentioned, nature has sources for various types of treatment, providing effective antimicrobials to treat infections caused by bacteria, viruses and fungi, while offering therapeutic support to multiple organs and body systems. On account of this, in this present research, we also do not rule out the possibility that lemons balm EE have interfered in aspects directly related to inflammation, since it possesses potential anti-inflammatory activities, supporting the traditional application of this plant in treating various diseases associated with inflammation and pain in more peripheral areas (Bounihi et al., 2013) and brain areas, whose active principles can probably be carried through the cerebral blood flow and interact with several neurotransmitters and signalling pathways involving kinase enzymes (Wightman, 2017).

Conclusion

Although, there is a wide variety of a drug to treat microbial infections, it is important to consider the frequency and severity of adverse effects. Moreover, as the arsenal of available antibiotics in the pharmaceutical market is increasingly being depleted due to microbial resistance, there is a need for more alternative and less toxic treatments. Thus, considering the results obtained here, it is possible to suggest that the extract from lemon balm can be considered a potential alternative for the treatment of diseases, which have similar (or no similar) reliability as conventional drugs DZP and FLX.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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