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Original Article

Long-Lasting Cognitive-Affective Disorders Induced In Rats After Neonatal Exposure to LPS: The Neuroprotective Effects of *Costus afer* (Costaceae) Extract

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ABSTRACT – *We aimed to assess the possible effects of hydroethanolic extract of Costus afer on long-lasting cognitive and affective disorders due to an early life neuroinflammation induction with lipopolysaccharide (LPS). At postnatal day 14(PND 14), neonate rats were exposed with single dose to LPS (250 µg/kg, i.p.), and 24 h later treated with either Costus afer extract (400 mg/kg, p.o.) or a reference drug melatonin (10 mg/kg, i.p) for two weeks. At PND 90, cognitive abilities as well as affective status were examined, followed by the biomarkers assay in the brain. As results, the Costus afer extract significantly prevented the neonatal exposure to LPS-induced recognition and working spatial memory deficits in adulthood. Furthermore, the extract of Costus afer attenuated anxiety- and depressive-like behaviors caused by the LPS. Otherwise, the treatment with Costus afer extract significantly inhibited the lipide membrane peroxidation through a reduction of malondialdehyde (MDA) level as well as the decrease of nitric oxide (NO) content, mainly in the hippocampus. Overall, the treatment with Costus afer extract seems to be a bit more efficient than that of the melatonin. Our findings suggest that Costus afer possesses some neuroprotective effects.*

However, additional pharmacological approaches are needed before promising Costus afer as effective therapeutic.

Keywords - *Anti-oxidative stress; cognitive-affective disorders; Costus afer; neonatal neuroinflammation*

I. INTRODUCTION

Most of the cognitive and behavioral impairments are resulting from neuroinflammatory processes. The chronic neuroinflammation is suggested to promote neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD)[1-3] as well as neuropsychiatric disorders including depressive mood. [4-5] In fact, AD is characterized by extracellular β-amyloid (Aβ) aggregated, extensive neuronal loss in the brain followed by memory deficit and cognitive decline. Previous researches have demonstrated a causal relationship of neuroinflammation as etiology factor in the occurrence of AD-like model of neuronal pathology in the brain.[6-7] Moreover, there is growing of evidence that the systemic inflammation is associated with spatial learning and memory, and cognitive impairments[8-9], whereas some other studies have revealed that the pathogenesis of depression are tightly linked to the neuroinflammation.[10] Neuroinflammation processes involve the activation of brain immune cells, namely microglia, which in turn release pro-inflammatory mediators like tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and contribute to free radicals generation.[11] One of the subsequent effects of neuroinflammation-mediated microglia activation is the production of nitric oxide (NO) catalyzed by *inducible*-nitric oxide synthase (*i*-NOS).

The administration of endotoxin lipopolysaccharide (LPS), a component of the outer membrane of negative-gram bacteria, is used as an experimental model of inflammation after intraperitoneal injection. In fact, LPS can induce neuroinflammation using rodents.[12] On the other hand, the systemic inflammation by the LPS could be accompanied by the production of the high level of blood cytokines released in the brain to cause neuroinflammation.[13] Also, it has been reported that LPS inters in brain tissue via the circumventricular and promotes locally the cytokines release to exacerbate the neuroinflammatory effects. Importantly, the brain of neonates is more sensitive in response to systemic

inflammation, and early life immune activation by LPS could have a short-term effect such as neurodevelopmental disturbance and long-term neurofunctional deficits. For instance, the immune activation between 3rd and 5th postnatal days increased the cytokines release in the hippocampus in adulthood [14] as well as cognitive impairments. [15]

Costus afer (Costaceae) is a rhizomatous herb of the Costus genus, mainly found in forest belts in the countries of West Africa and in South Africa [16]. It is used as folklore medicine to treat and manage a range of diseases, including malaria, diabetes mellitus, arthritis, stomach disorders, inflammation. [17] *Costus afer* is rich-polyphenol compounds, and the phytochemicals constituents are essentially represented by flavonoid and alkaloid.[18] Also, it has been reported some anti-inflammatory properties of *Costus afer* using a rat's model of Arthritis [19], as well as antioxidant and lipid peroxidation inhibition properties. [18] However, no study has been led on the neuroprotective actions of *Costus afer* against LPS-induced neuroinflammation and the subsequent neurofunctional alterations. The basis of this, in the present study, we aimed to examine the effects of hydroethanolic extract of *Costus afer* on a rat's model of early life neuroinflammation due to LPS exposure and long-lasting cognitive-behavioral dysfunctions.

II. MATERIAL AND METHODS

A. Animals

The experiment was carried out using Wistar rats strain obtained at the vivarium of normal superior School (ENS) of Abidjan, Ivory Coast. The pregnant rats were kept in acclimation under normal conditions of air temperature at 22 ± 2ºC, relative good humidity 60%, and on a 12-h light /12-h dark cycle. They had access to tap water and food *ad libitum.* All experimental protocols were carried out according to the NIH guide for the care and use of laboratory animals in order to minimize the number of animals used and their suffering.

B. Experimental procedure

The experimental model was performed according to Berkiks et *al.* [20] with some slight modifications. At the PND 14, the pups were separated briefly from their mams and were injected either with NaCl (0,9%, i.p.) or 250µg/ kg, i.p of LPS (Merck, Morocco). Twenty-four hours later, some LPS injected rats were treated either with melatonin (10 mg/kg, i.p.) or *Costus afer* crude extract (400 mg/kg, p.o.) for 14 consecutive days. After all the treatments, animals were carefully followed up PND 90 before cognitive-behavioral testing.

The dose of 2000 mg/kg p.o. of *Costus afer* was considered safe according to OECD recommendation. [19]

C. Behavioral analysis

a) Elevated Plus Maze (EPM)

The EPM is a behavioral test widely used to study an animal's model of anxiety induced by an anxiogenic substance. [21] The apparatus consisted of four arms arranged in the form of the cross. Two open arms (25-cm height cm x 10 cm width) as opposed to two enclosed arms (40-cm walls), with an intersection as a common central platform (5x5 cm). The EPM was raised to 50 cm above the floor and lighted with a halogen lamp of 9lx. The test began by placing the animal onto the central square facing an open arm and allowing 5 min to explore freely. The time spent inside open arms was computed to evaluate anxiety-related behavior. The apparatus was cleaned with ethanol 10% between tests.

b) Forced swimming test (FST)

The FST was performed to evaluate depression-like behavior as described firstly by Porsolt et *al.*[22] It's based on the assumption that when placing an animal in a container filled with water, it will first make efforts to escape but eventually will exhibit immobility that may be considered to reflect a measure of behavioral despair. This study was realized with some modifications. There was one 6 min-session divided into pre-test (the first 2 min) and test (the last 4 min). Fill before the cylinders with tap water at 25°C and adjust the water depth according to the mouse's size so that it cannot touch the bottom of the container with its hind legs. Then turn on the video camera placed on the level of the water surface to clearly appreciate active and passive behaviors and then place each mouse in the water-filled cylinder container for 6 min. After time had elapsed, we turned off the camera, removed the mouse from the container, and placed it in the transient drying cage with the heat lamp above it and the heat pad under it. We changed the water after every session to avoid any influence on the next mouse. The stillness posture is characterized by floating in the water with only the movements necessary to keep the nose above the surface. The time of immobility was recorded.

c) Y-maze

This test was performed to evaluate spatial working memory in rodents after all the treatments. [23] The apparatus was made in fine wood with three arms (A, B, and C) measuring 40 in length, 10cm in width, and 13 cm in height) and painted in different color patterns. A central platform is formed by triangles of 120° between each arm. The procedure consists of giving 8 min-session to each rat for exploring the maze freely. The sequence of arms entries was monitored with a camera video. An entry is validated when the four paws are within the arms. Alternation is defined as a triad of successive entries in different arms (*i.e.*, ABCACBAACB= 5 alternation). Spontaneous alternation behaviour was calculated with the following equation: % alternation = 100 x (number of alternation/ total arm entries – 2).

d) Recognition Memory Test (NORT)

The object recognition test procedure was conducted as described by Ennaceur and Delacour. [24] The apparatus is an Open box with floor measurements 50 cm in length, 50 cm in

width, and 40 cm in height walls. In the trial (familiarization session), rats were allowed 5 min to explore the box freely with two identical objects and return to their home cage. After 2 h delay, to evaluate short-term recognition Memory (STM), rats were returned to an open field in which one object was switched by another one different for color, shape, and size, and the experiment was repeated for 5 min with one novel object and one identical previously explored. To evaluate long-term memory (LTM) 24h later from the familiarization phase, rats were submitted to explore two objects again for 5 min, one identical and another novel one. Objects and boxes were cleaned with ethanol 70% during the intertrial period. The exploration time of each object was recorded the video tracking, and the exploration feature is defined as directing the nose at a distance less than 1 cm from the object. The ratio of preference of the novel object of each animal was calculated from the exploration frequency of the novel object divided by the total frequency spent for exploring both objects.

D. Biochemicals assay

a) Tissues preparation

Twenty-four hours after the behavioral tests, the mice are euthanized with 7% chloral. Then, hippocampal and PFC cortex tissues from each rat were isolated, weighed, and homogenized in ice-cool 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm/min for 10 min. The supernatant (S1) was collected and kept at -20°C till the assay day.

b) Nitrite oxide (NO) content

The quantification of NO content was determined by nitrite level in a sample using the modified Griess method.[25] Briefly, the supernatant S1 was mixed with 50µl of Griess reagent (1%) sulphanylamide (A) and 0.1% N-1-naphthyl ethylenediamine dihydrochloride (B) in 2.5% orthophosphoric acid)], and the mixture was incubated at 37°C for 10 min in the dark. The reaction was performed in two steps. The first one consisted of a denitrogenation reaction between the nitrite and Griess reagent A, leading to a Diazonium salt by-product. The second step is the formation of a stable chromophoric Azo product resulting from the coupling between Griess reagent B and the Diazonium salt. The Azo product strongly absorbs at 543 nm at the ELISA reader. The NO concentration was expressed in µmol/g of tissue.

c) Determination of lipid peroxidation level

The assay Malondialdehyde (MDA) level, an important index of lipid peroxidation, was described in the method of Satoh et *al*.[26] Briefly, supernatant S1 was mixed with 1.5 ml of trichloroacetic acid (10%), vortexed, and incubated at room temperature for 10 min. Then it was added to the mixture 1,5 ml of thiobarbituric acid (0.67%) and heated in boiling bath water for 15 min. After cooling, 1.5 ml of n-butanol was mixed into the solution and strictly vortexed. The sample was centrifuged at 800 rpm for 5 min, and the supernatant S2 was collected. The absorbance was determined spectrophotometrically at 532 nm. The results were expressed as MDA level in µmol/g of tissue.

E. Statistical analysis

GraphPad Prism 6.0 version was used to record data and their analysis. The experimental results data were expressed as mean ± S.E.M (Standard Error of Mean). Statistical analysis was done one-way analyses of variance followed by Tukey *post-hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSION

A. Effects Costus afer extract on Cognitive-affective status

a) The effects of Costus afer extract on LPS-induced anxiety-like behavior

In the EPM, the percent of time spent in open arms was significantly reduced in rats of the LPS group ($P < 0.01$) when compared to the PBS group. However, the treatment of LPS rats with *Costus afer* extract improved as well as with melatonin the % of time spent in open arms, but not significantly (Fig 1). It suggests that the extract of *Costus afer* could have a bit anxiolytic effect

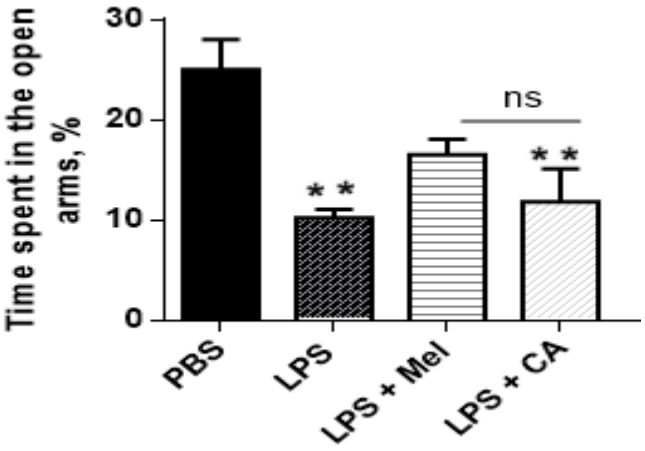

Fig. 1 The effect of *Costus afer* extract on the anxiety-like behavior level in adult rats early-life exposed to LPS percent of time spent in the open arms. Data are expressed as mean ± SEM. One-way ANOVA / Tukey post hoc analysis. ** $P < 0.01$ (vs. PBS group); ns, non-significant

b) The effect of Costus afer extract on LPS-induced depression-like behavior

In the FST, the immobility time in the LPS group of rats was increased without any significant difference in comparison to the groups of rats LPS-treated with *Costus afer* extract and PBS. However, the immobility time was significantly increased in LPS rats treated with melatonin ($P < 0.05$) (Fig

2). It suggests that *Costus afer* could possess some anti-depressant properties.

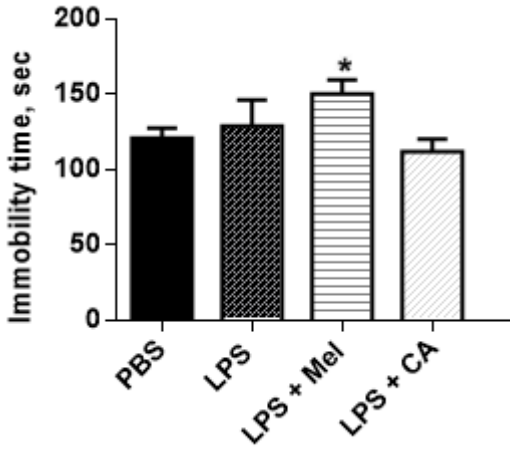

Fig. 2 The effect of *Costus afer* extract on the anxiety-like behavior level in adult rats early-life exposed to LPS
Immobility time in FST. Data are expressed as mean ± SEM. One-way ANOVA / Tukey post hoc analysis. * *P* < 0.05 (LPS + Mel vs. other experimental groups)

c) The effect of Costus afer extract on LPS-induced working spatial memory alterations

As shown in Fig.3, there was a significant alteration of working spatial memory in the LPS group of rats. In fact, the percent of alternation in behavior was significantly reduced in LPS rats compared with those of PBS rats ($P < 0.001$), LPS-treated melatonin rats ($P < 0.05$), and LPS-treated with *Costus afer* extract ($P < 0.01$).

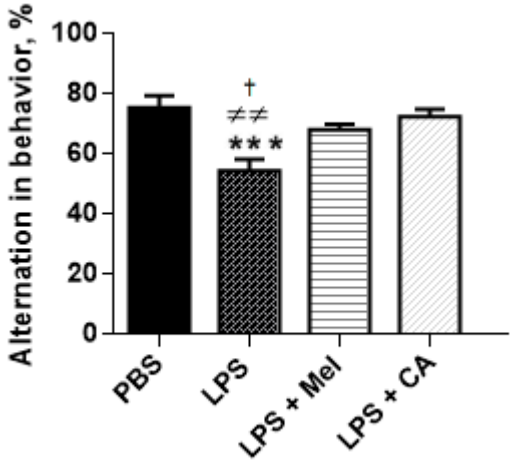

Fig. 3 The effect of *Costus afer* extract on the spatial working memory abilities in adult rats early-life exposed to LPS
Percent in behavior alternation in Y-maze. Data are expressed as mean ± SEM. One way ANOVA / Tukey post hoc analysis. * *P* < 0.001 (LPS group vs. PBS), ≠≠ *P* < 0.01 (LPS vs. LPS + Mel), † *P* < 0.05 (LPS vs. LPS + CA)**

d) The effects of Costus afer extract on LPS-induced recognition memory alterations

As depicted in Fig 4, the recognition index was below the threshold of recognition (50%) in the LPS rats. We found that there was no significant difference regarding the index of the short-term recognition memory between the experimental groups, even if the treatment of LPS rats with *Costus afer* extract slightly improved the index (Fig 4a). By contrast, the long-term recognition was significantly impaired in LPS rats through the reduction of the recognition index ($P < 0.001$ vs. other groups). Interestingly, the treatment with LPS rats *Costus afer* extract demonstrated a significant increase of the long-term recognition index, which is extensively above the threshold of recognition compared to that melatonin ($P < 0.01$) (Fig 4b). Taken together, these results suggest that the long-term memory capacities are more sensitive to early-life exposure to LPS when evaluating in the adulthood stage, but *Costus afer* compounds act efficiently as the remedy.

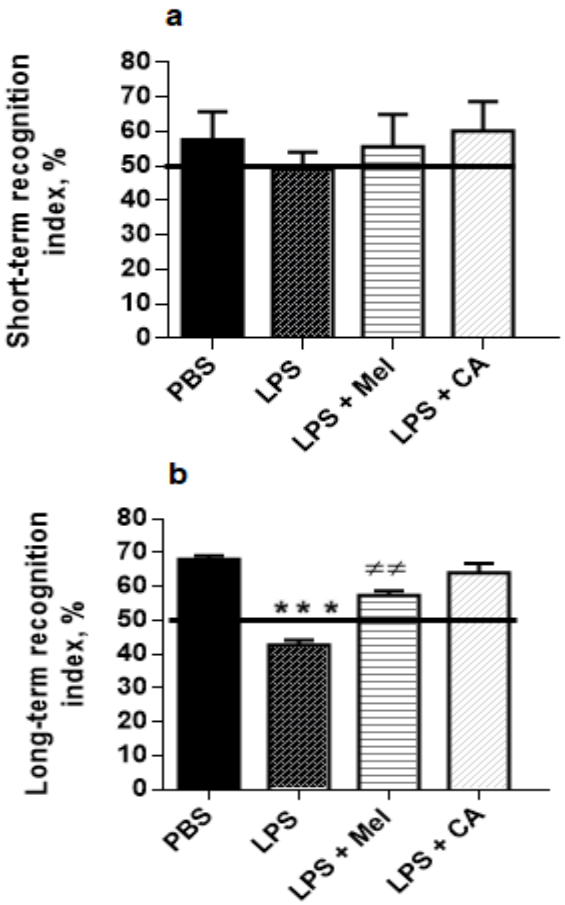

Fig.4 The effect of *Costus afer* extract on the recognition memory abilities in adult rats early-life exposed to LPS
Short-term recognition index (a); Long-term recognition index (b). Data are expressed as mean ± SEM. One way ANOVA / Tukey post hoc analysis. * *P* < 0.001 (LPS group vs. other experimental groups), ≠≠ *P* < 0.01 (LPS + Mel vs. LPS + CA)**

B. Oxidative stress markers

a) The effects of Costus afer extract on LPS-induced NO content change

In the hippocampus, we found that the NO level was highly increased in the LPS group of rats ($P < 0.001$) when compared to other studied groups. In addition, the NO content was significantly increased in LPS rats treated with *Costus afer* extract ($P < 0.01$) compared to those treated with melatonin.

In the prefrontal cortex area, the level of NO was also significantly increased in LPS treated rats ($P < 0.001$), but the treatment with *Costus afer* extract didn't the NO content ($P < 0.001$) when compared to that of melatonin (Fig 5).

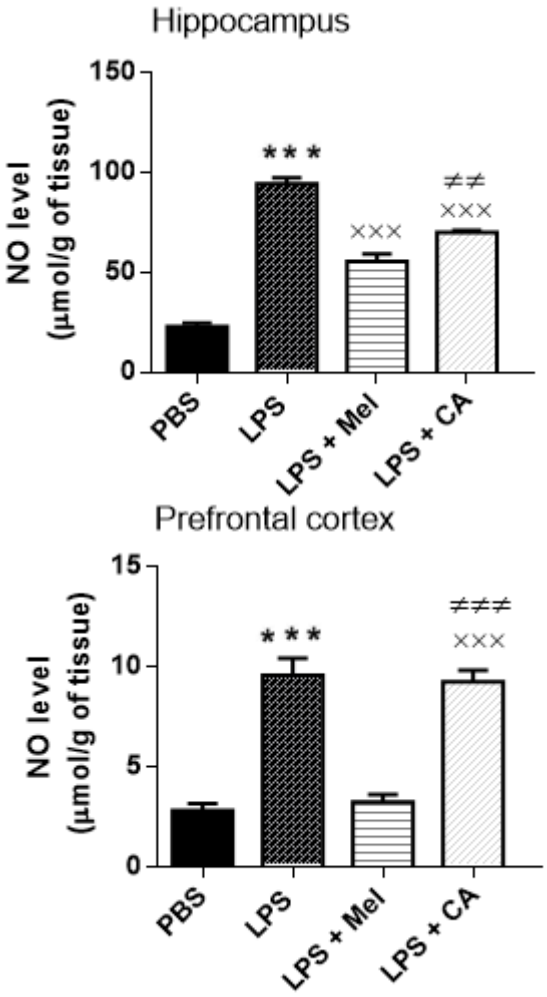

Fig.5 The effect of *Costus afer* extract on the NO content in adult rats early-life exposed to LPS
NO content expressed in the hippocampus and the prefrontal cortex. Data are expressed as mean ± SEM. One way ANOVA / Tukey post hoc analysis. *** $P < 0.001$ (LPS group vs. PBS), ^{xxx} $P < 0.001$ (LPS group vs. LPS + Mel or LPS + CA), $^{\neq\neq}P < 0.01$, $^{\neq\neq\neq}P < 0.001$ (LPS + Mel vs. LPS + CA)

b) The effects of Costus afer extract on LPS-induced MDA level change

The LPS caused significant lipid membrane peroxidation. In the hippocampus, the level of the end-product MDA was significantly higher in LPS rats ($P < 0.001$) compared to PBS groups. *Costus afer* treatment of LPS rats significantly mitigated the MDA level ($P < 0.05$).

In the prefrontal cortex, we also reported a highly significant level of MDA ($P < 0.001$) in LPS rats compared to PBS rats; however, it was highly significantly reduced in LPS rats treated with *Costus afer* ($P < 0.001$) and lesser with the melatonin treatment ($P < 0,05$) (Fig 6).

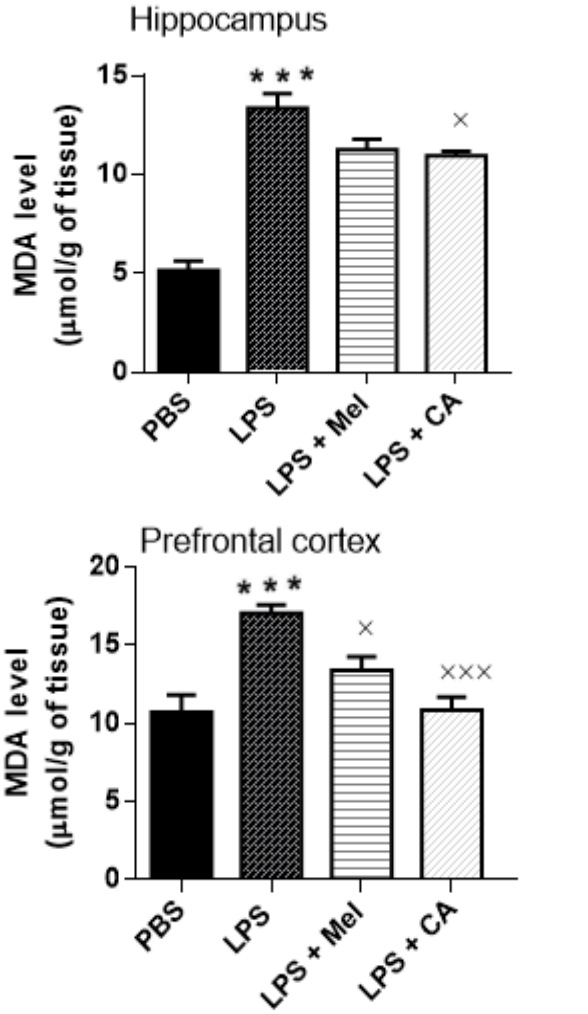

Fig. 6 The effect of *Costus afer* extract on the lipid membrane peroxidation level in adult rats early-life exposed to LPS
MDA level expressed in the hippocampus and the prefrontal cortex. Data are expressed as mean ± SEM. One way ANOVA / Tukey post hoc analysis. *** $P < 0.001$ (LPS group vs. PBS), ^{xx} $P < 0.01$, ^{xxx} $P < 0.001$ (LPS group vs. LPS + Mel or LPS + CA).

To our knowledge, this is the first study examining the neuroprotective effects of *Costus afer* extract on long-term cognitive-behavioral impairments after neonatal exposure to LPS. Briefly, our results showed that the adulthood cognitive-behavioral deficits related to neonatal LPS-induced systemic inflammation are associated with changes in inflammatory biomarkers such as the increase of NO production and lipide peroxidation level in both hippocampal and prefrontal cortex areas. By contrast, the treatment with *Costus afer* extract attenuated working spatial memory and recognition memory alterations, as well as anxiety -and depression-like behaviors level following the LPS-induced early life neuroinflammation. Also, the *Costus afer* extract administration has significantly reduced LPS-induced neuroinflammation and the subsequent biomarkers release.

Previous studies reported that an injection of LPS early during the development led to affective behavior alteration, including depression or anxiety at the adult stage. [20, 27] The behavioral disorders were associated with an elevation of pro-inflammatory markers such as TNF-α and NO, which resulted in the overactivation of microglial cells in the hippocampus. In fact, neuroinflammation is considered an important pathogenesis factor in which the release of cytokines causes general behavioral depression. [28] It has also been suggested that TNF-α can promote the activity of indolamine 2,3-dioxygenase (IDO) by an interferon-γ (IFN-γ) independent mechanism.[29] In addition, previous studies revealed that cytokines such as IFN- γ, and TNF-α are capable of activating necrosis factors kappa B (NFkB) and IDO efficiently.[29, 30] IDO, a rate-limiting extrahepatic enzyme, is involved in tryptophan catabolism via the kynurenine pathway during the chronic inflammation condition and ensures a thigh relationship between brain immune cells activation and behavioral responses. A disordered activation of IDO leads to excessive depletion of tryptophan and consequently the development of depression symptoms. [10, 31] Also, it is well known that tryptophan is the amino acid precursor of serotonin synthesis, which is the regulator au depression as well anxiety levels. Otherwise, we reported significant impairment of working spatial memory (evaluated with Y-maze) and recognition memory (evaluated with NOR test) in LPS rats, as well as the high level of neuroinflammation markers in the hippocampus. Although the mechanisms underlying those memory deficits remain unclear, it has been suggested that inflammation-induced chronic cytokines upregulation by microglia cells activated plays a critical role.[32, 33] A previous report mentioned that the long-term effect of LPS -induced high levels of pro-inflammatory cytokines such as TNF-α, IL-1β, or IL-6 are potentially associated with hippocampal neurons dysfunction. [34] Interestingly, Deng et *al.* [35] have demonstrated that the LPS-induced chronic neuroinflammation caused an occurrence of AD-like amyloidogenic axonal pathology and dendritic degeneration. Even if, in the current study, we didn't assess the levels of pro-inflammatory cytokines nor the microglial cells overactivation, we have reported a significant NO content in the hippocampus and the prefrontal cortex of LPS rats. This has also been the finding of Berkiks et *al.* [21] when studying the long-term behavioral effects of neonatal (PND 14) exposure to LPS. The activated microglial cells in LPS-induced inflammation enable the up-regulation of iNOS expression. [36] The resulting in excessive NO production contributes to oxidative stress leading to neuronal damage or death. [37]

The coadministration of melatonin to neonate rats (PND 7) injected with LPS significantly inhibited the neuroinflammation, ROS generation, oxidative stress damage, apoptotic neurodegeneration in the dentate gyrus hippocampus.[38] Melatonin is known to be a potent antioxidant due to its ability to free radical scavenging, to prevent oxidative stress, and to inhibit neuroinflammation in multiple models [39,40], including LPS. [41] For this reason, in the present study, the neuroprotective effects of *Costus afer* extract have been compared to melatonin, as a reference drug. Our results showed that *Costus afer* extract treatment counteracted significantly LPS-induced neuroinflammation and cognitive impairment long-term after its induction. The treatment with *Costus afer* extract showed more beneficial effects on altered working spatial memory, spatial learning, and recognition memory abilities found in the LPS group of rats, compared to melatonin. Also, the effects of *Costus afer* extract on LPS-induced affective disorders were positive but less pronounced when compared to LPS rats without treatment. The effective effects of *Costus afer* extract on the cognitive-behavioral changes could be attributed to its flavonoid-rich compound, the anti-antioxidant [18], and anti-inflammatory properties. [19] The neuroprotective effects of *Costus afer* seem to be manifested by the prevention of ROS actions and the repression of pro-inflammatory markers since we found a significant lipid membrane peroxidation inhibition (decrease in the MDA level) as well as a decrease of NO production in the brain of LPS rats treated with *Costus afer* extract. Our results are in agreement with those of Berkiks et *al.* [20], which indicated that *Thymelaea lythroides* strongly reduced the NO production and the MDA level in the hippocampus of LPS rats exposed at PND 14. In fact, the LPS generates ROS, which in turn induces acute neuroinflammation [42,43] *via* the activation of NFkB, the increase pro-inflammatory responses of iNOS and cyclooxygenase 2 (COX 2) at both mRNA and protein levels. [44] On the other hand, the flavonoid contained in *Costus afer* could have some neuroprotective effects through the neuronal or microglial signaling pathway involved in the reinforcement of neuronal capacity to counteract prooxidant and pro-inflammatory mediators. [45] Taking together these analyses, we can suggest that *Costus afer* extract may be an effective approach to attenuate neonatal LPS-induced long-lasting cognitive and affective disorders, as well as neuroinflammation in adulthood. Nevertheless, the part of alkaloid presents in *Costus afer* as neuroprotector compound

against LPS-neonatal neuroinflammation remains to be elucidated.

IV. CONCLUSION

In conclusion, the administration of *Costus afer* extract significantly relieved cognitive-behavioral disorders in rats that were exposed to LPS during the neonatal stage. *Costus afer* exhibited both anti-inflammatory and antioxidant properties against the LPS-induced early life immune system activation leading to long-lasting chronic neuroinflammation and, therefore, brain disorders

V. ACKNOWLEDGMENT

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