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Preliminary Study on the *In vivo* Anti-neuroinflammatory Effects of *Khaya grandifoliola* and *Cymbopogon citratus* Polysaccharide Fractions

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KFM, TB and PY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author GM managed the analyses of the study. Author KFM also managed the literature search. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the effects of polysaccharide fractions named KGF and CCF respectively for *Khaya grandifoliola* stem bark and *Cymbopogon citratus* leaves on Central Nervous System (CNS) depression and on systemic lipopolysaccharide (LPS)-induced brain inflammation and hyperalgesia in BALB/c.
Methodology: BALB/c mice weighing about 25-35 g were used for the experimentation. Depressant effects of polysaccharide fractions were firstly evaluated using Rota Rod and Actophotometer apparatus. Secondly, LPS or saline solution (5 mg/kg) was Intraperitoneally administered (*i.p.*) 1 hour after oral administration of polysaccharide fractions (100 mg/kg test dose, *p.o.*) or

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distilledwater. Then, the hot plate and tail-flick models were performed 1 hour after LPS injection to determine thermal hyperalgesia and brain inflammation, was examined 3 hours after LPS injection by Luminex assay.
Results: Systemic LPS administration resulted in a reduction of pain response latency and an increasing expression of nuclear factor-κB (NF-κB) and pro-inflammatory cytokines interleukin-1β (IL-1β), IL-6, tumor necrosis factor- α (TNF-α) genes in brain after 24 hours. From the results it was observed that treatment with KGF and CCF (100 mg/kg, *p.o*) significantly attenuated LPS-induced hyperalgesia and overexpression of brain levels of IL-1β, IL-6 and TNF-α genes dependent on inhibition of the NF-κB signaling pathway in BALB/c without CNS depressant properties.
Conclusion: The present findings confirm the potential of KGF and CCF in the treatment of neuroinflammation-related diseases and it warrant further testing for the development of a new chemical entities. However further studies are required for determination of effective dose and mechanism of action associated.

Keywords: Polysaccharides; Khaya grandifoliola; Cymbopogon citratus; anti-neuroinflammation; In vivo.

1. INTRODUCTION

Inflammation is a physiological response to injury that is designed to remove dangerous stimuli, kill bacteria, remove cell debris, and initiating healing [1]. The immune system and brain communicate and attacks on the peripheral immune activation can also induce inflammation in CNS known as neuroinflammation by using several neural and humorous pathways. Neuroinflammation is mediated by several pro-inflammatory cytokines including Interleukin-1β (IL-1 β), IL-6 and Tumor Necrosis Factor (TNF-α), chemokines and secondary messengers (Reactive Oxygen Species, [ROS], Nitric Oxide [NO]) mainly produced by activated resident CNS cells (glial cells) microglia and astrocytes [2]. Glial cells are also strongly associated with the sensory nervous system and neuroinflammation can give rise to neuropathic pain [3-4]. In fact, combined activation of microglia and astrocytes, and the production of cytokines such as IL-1 and TNF, cause a change in the signaling of the astrocyte network, thus potentiating neuronal pain transmission [2]. Neuroinflammation is a beneficial response, highly regulated and rapidly resolved. Nevertheless, it may persist even after elimination of the initial stimulus and give rise to a highly destructive and pathological neuro-inflammation associated with excessive activation of glial cells with significant production of cytokines [2-5]. Consequently, neuroinflammation is a common component of the pathogenesis for cerebral malaria, cerebral tumor and multiple neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, Multiple sclerosis and Amyotrophic lateral sclerosis [6-8]. In order to study the neuroinflammatory processes of these diseases, it was necessary to create *in vitro* and *in vivo* models that reproduced as faithfully as possible the manifestations found in humans. In recent years, LPS has been one of the most widely used toxins. In addition, it is one of the most powerful activators of glial cells. According to [6,9-10], peripheral inflammation induced in adult animals by systemic injection of LPS (5 mg/kg, intraperitoneal) leads to a rapid increase in the production of TNF-α in-brain (high content for 10 months) leading to the activation of brain microglia for chronic production of brain pro-inflammatory factors such as TNF, IL-1, NF-κB p65 and NO. Anti-inflammatory treatments are used in addition to specific treatment of each case of diseases to relive pain and inhibits chronic inflammatory processes [11]. However due to the potential secondary effects of anti-inflammatory drugs commercialized (teratogenic, mutagenic, cancerigenic, gastric, metabolic, endocrinal, neuronal disorders), it is necessary to opt for the search of much safe and new active compounds. Thus, phytotherapy is a source of natural bioactive molecules that can be used for treatment of inflammation-related diseases [12]. *Khaya grandifoliola*(Meliaceae) and *Cymbopogon citratus* (Poaceae) are two plants of Cameroon pharmacopeia, used for the treatment of malaria, rheumatism and infectious diseases that provoke fever, pain and inflammation. Thus, numerous studies have highlighted biological activities of promising compounds in their extracts among which flavonoids, alkaloid and polysaccharides, thus justifying their use in the treatment of inflammatory related-diseases [13-17]. Our previous work in [18-19] aimed to explore the effect of low and high molecular weight polysaccharides of both plants on

neuroinflammation. A model of LPS-induced inflammation injury in U87-MG glioblastoma cells was constructed. We found that low molecular weight polysaccharide fractions named KGF and CCF respectively for *K. grandifoliola* and *C. citratus* have no effect on U87-MG cells viability up to 100 µg/mL, but mostly attenuate LPS-induced impairment of U87 cells. The protective functions of the two polysaccharide fractions on U87 cells are through down regulating Reactive Oxygen Species (ROS) overproduction, suppressing TNF-α, IL-6 and IL-1β pro-inflammatory cytokines expression dependent on inhibition of the NF-κB signaling pathway [18-19]. These *in vitro* findings suggested that KGF and CCF possibly acted as potential therapeutic drugs for treatment of neuroinflammation-related diseases. These results are very encouraging, however, the role of KGF and CCF in neuroinflammatory response on *in vivo* models has not been elucidated. Hence, the present work was intended to study their effects on systemic LPS-induced pain sensitivity and acute brain inflammation in BALB/c mice.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Forty-six male (08 weeks old) BALB/c mice weighing about 25-35 g were supplied by animal house of the Birla Institute of Technology and Science (BITS)-Pilani, Hyderabad campus, Jawahar Nagar, Hyderabad–500078, Andhra Pradesh, India. The animals were kept in polypropylene cages with metal mesh cover to acclimatize at an ambient temperature of 20°C ± 3°C, 12 hours light/dark cycle and adequate ventilation was given for 07 days. Then, standard food and running water were given *ad Libitum*. The experiments were carried out during the light period (9.00–16.00 hours). Each group consisted of six animals and the dose which was oral administered to experimental animal was based on the literature of acute toxicological studies of plant polysaccharides [20].

2.2 Preparation of Polysaccharide Fractions

Khaya grandifoliola C.D.C. (Welw) (Meliaceae family) stem Bark and *Cymbopogon citratus* Stapf (Poaceae family) leaves were harvested at Mbalmayo forest and Emana respectively (Centre region, Cameroon). After their identification at the National Herbarium of Cameroon (Voucher specimen N°52658/SFR and 14243/HNC respectively for *K. grandifoliola* and *C. citratus*), low molecular weight polysaccharides KGF (*K. grandifoliola*) and CCF (*C. citratus*) were isolated as described by our previous studies [19]. Briefly, the plant material were defatted and de-colored using methanol. Then, KGF and CCF were obtained from the resulting material by boiling in water, precipitation in two volumes of ethanol, deproteinization using Sevag reagent, dialysis and freeze-drying.

2.3 Performance and Motor Coordination Activity

The motor coordination and performance of each mouse was evaluated 60 minutes after polysaccharide fractions treatment (100 mg/kg, *p.o.*) in Rota-rod apparatus (Dolphin^{TM}.) This equipment has a bar 2.5 cm in diameter and divided into three parts, and it is placed at a height of 50 cm, rotating at 20 rpm. Latency to fall from the rotating bar during 5 minutes was registered. Finally, reduction in fall-of-time percentage corresponding to the percentage decrease in performance and motor coordination activity was determined [21].

2.4 Performance and Motor Coordination Activity

The locomotor activity can be easily studied with the help of digital actophotometer (Dolphin^{TM}.) Each animal was placed individually and the basal activity score of all the animals were recorded after 60 minutes of KGF or CCF (100 mg/kg, *p.o.*) treatment, and distilled water was used as vehicle. Then, the activity on each mouse was tested for 5 minutes. The difference in the activity was recorded considering before treatment values and after treatment values. Finally, reduction in locomotor activity percentage was determined [21-22].

2.5 Induction of Acute Neuroinflammation by LPS

Lipopolysaccharide (LPS) from E.coli serotype 0111:B4 purified by phenol extraction was purchased from Sigma Aldrich. Mice were divided into four groups as follow:

- **Group one** (Naive) consisted of non-treated mice receiving oral administration of distilled water one hour before single intraperitoneal injection of 0.9% NaCl solution.

- **Group two** (LPS) consisted of mice treated with 5 mg/kg of LPS (prepared in 0.9% NaCl solution) by single intraperitoneal injection (*i.p.*).

- **Group three** and **Group four** (KGF &CCF) consisted of mice treated with 100 mg/kg (*p.o.*) of KGF or CCF polysaccharides, one hour before *i.p.* injection of 5 mg/kg of LPS.

2.6 Tail-flick Test

The tail-flick test is designed to assess the thermal nociceptive threshold by using hot water (45 ± 1°C.) The tail-flick test was performed as described in [23].with modification at time zero (0 minutes) and 60 minutes after LPS or saline solution injection. The lower 5 cm portion of each tail was immersed in a beaker of hot water. The pain sensitivity was measured by tail -flick latency, which is defined as the time from the onset of thermal heat to tail withdrawal. A cut-off time of 20 sec set to minimize tissue damage. The percentage maximum possible effect, MPE was defined as the lack of a nociceptive response during 20 s (cut-off time) of exposure to the heat stimulus. MPE for these time points were determined using the formula:

$$\text{MPE}\ (\%) = \frac{(\text{Post treatment value}) - (\text{Pre} - \text{treatment value})}{(\text{Cut} - \text{off value}) - (\text{Pre} - \text{treatment})}$$

2.7 Hot Plate Test

This test is designed to assess the thermal nociceptive threshold by using a heated plate (55 ± 1°C.) Before experimental treatment, each mouse was dropped on the heated plate to familiarize the animal with the test procedure. The time of latency was defined as the time period between zero point when the animal is placed on the hot plate surface and the time when the animal licked its fore paw or jumped off to avoid thermal pain. Baseline latency was then determined for each mouse. The latencies of both fore paws licking or jumping were measured at time zero (0 minutes), 60 and 120 minutes after LPS or saline solution injection, with the cut-off time of 20 seconds [23].

2.8 Sample Collection

Three hours post LPS administration, mice were killed by spinal dislocation and the brain were removed from animals under aseptic conditions. Then, brain tissue of each mouse was conserved in -80°C for gene analysis.

2.9 Evaluation of Inhibitory Effect of Lps-induced Over expression of Pro-Inflammatory Cytokines

Three hours post saline or LPS administration, brain sample (100 mg) were collected and treated with 1 mL of Trizol according to manufacturer's instruction. RNA was then extracted, quantified with nanodrop and the purity confirmed as described by our previous studies [19]. RNA (1 µg) was reversed-transcribed using *Verso* cDNA synthesis kit (*Thermo scientific*, #AB-1453A) and quantitative Real Time PCR was performed on reversed-transcribed products for determination of inflammatory gene expression according to the manufacturer's instructions using a BIO-RAD CFX Connect, with SYBR Green (Kapa Biosystem) as the fluorescent dye, enabling real time detection of PCR products [19].

2.10 Statistical Analysis

Results were expressed as the means ± SEM. Multi group comparison was performed by one-way analysis of variance, followed by the Dunnett's multiple comparisons as a post hoc analysis test for comparison between polysaccharides treated groups, naive and LPS groups at P = .05, P = .01 and P = .001. Calculations were performed using GraphPad InStat® version 6.01 software.

3. RESULTS

3.1 Muscle Coordination and Locomotor Activity

In the motor coordination assay, *Khaya grandifoliola* and *Cymbopogon citratus* polysaccharide fractions (100 mg/kg, *p.o.*) slightly reduced after 60 minutes the time spent by the animals on the revolving rod when compared with Naive. Moreover, the reduction of locomotor activity of polysaccharides treated groups were 18.58% and 19.69%, slightly higher when compared to Naive group. However, the results were not significant statistically (P value not significant). It may be due to the non CNS depressant properties of KGF and CCF polysaccharides. The results are tabulated in Table 1 and Table 2.

Table 1. Effect of KGF and CCF on muscle coordination activity using Rota-rod

Treatments	Fall-off time Latency		
	Mean fall-off time before treatment	Mean fall-off timeAfter treatment	Reduction in Fall-of-time (%)
Naive	298.33 ± 0,61	297.5 ± 0.84	0.28
KGF	294.67 ± 2,98	292,17 ± 1.87	0.85
CCF	296 ± 3.27	295.5 ± 3.18	0.17

KGF-Polysaccharide fraction of Khaya grandifoliola, CCF- Polysaccharide fraction of Cymbopogon citratus. Polysaccharide fractions (100 mg/kg) were administered orally 60 minutes prior the test. Values represented mean ± SEM (n=6), P=.05. No difference between polysaccharide fractions treated and naive groups

Table 2. Effect of KGF and CCF on locomotor activity using Actophotometer

Treatments	Activity score		
	Mean activity score before treatment	Mean activity score after treatment	Reduction in Locomotor activity (%)
Naive	337.67 ± 10.67	275.33 ± 17.33	18.46
KGF	310.33 ± 20.67	241.67 ± 44.2	18.58
CCF	389.33 ± 5.33	312.67 ± 6.33	19.69

KGF-Polysaccharide fraction of Khaya grandifoliola, CCF- Polysaccharide fraction of Cymbopogon citratus. Polysaccharide fractions (100 mg/kg) were administered orally 60 minutes prior the test. Values represented mean ± SEM (n=6), P=.05. No difference between polysaccharide fractions treated and naive groups

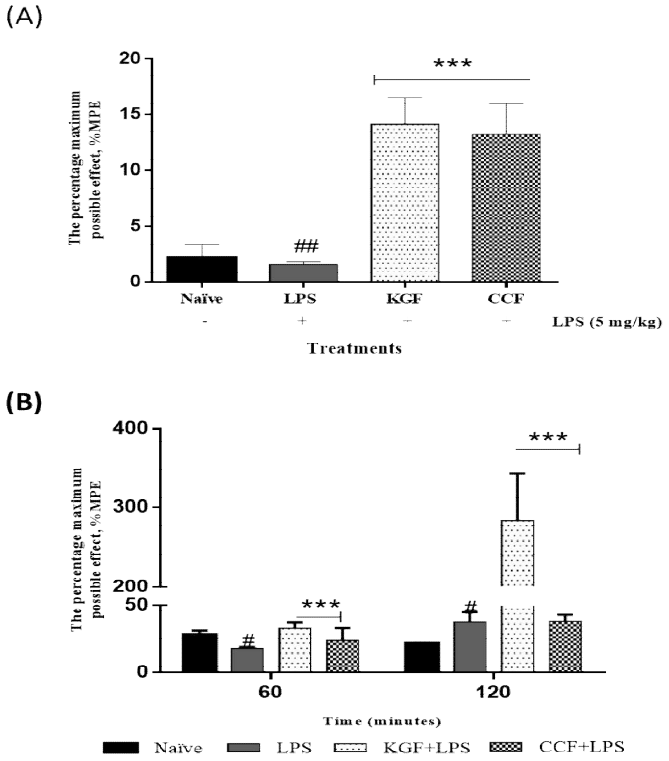

Fig. 1. KGF and CCF attenuated systemic LPS-induced hyperalgesia in BALB/c mice in tail-flick (A) and hot plate (B) tests

*Naive-saline solution treated mice; LPS- saline solution + LPS treated (5mg/kg, i.p.) mice, KGF & CCF - K. grandifoliola and C. citratus polysaccharide fractions (100 mg/kg, p.o.) + LPS (5 mg/kg, i.p.) treated mice. Data are expressed as mean ± SEM. *, **, *** denote statistical significance at p<0.05, p<0.01 & p<0.001 in comparison to the LPS group and #, ##, ### denote statistical significance at p<0.05, p<0.01 & p<0.001 in comparison to the naive group.Mean ± S.E.M = Mean values ± Standard error of means of six experiments*

3.2 KGF and CCF Attenuated Systemic LPS-Induced Thermal Hyperalgesia

Fig. 1 shows the effect of systemic LPS exposure on latency time in the tail flick (Fig. 1.A.) and the hot plate (Fig. 1.B.) tests in the BALB/c mice, with or without polysaccharide fractions of *K. grandifoliola* and *C. citratus*. One hour after LPS or saline solution injection, BALB/c mice in the LPS group showed a significant decrease in the mean latency in the tail-flick and hot plate tests compared with BALB/c mice in the Naive group (*P*=.01, *P*=.05). This decreased removal latency (i.e., an enhanced response to stimuli) is characteristic of hyperalgesia in BALB/c mice. However, two hours after LPS or saline solution injection there is no difference between Naive and LPS groups (Fig. 1.B.) KGF and CCF polysaccharides treatment one hour before LPS injection effectively prevent (*P*=.001) systemic LPS-induced pain hypersensitivity in BALB/c (Fig. 1.A & Fig. 1.B).

3.3 KGF and CCF Attenuated Systemic LPS-Induced Inflammatory Responses in Brain

Three hours following LPS or saline injection, there were no significant effect of LPS on peripheral damage but inflammatory gene expressions were found altered. In fact, as shows in Fig. 2, Interleukin-6 (IL-6), IL-1β, Tumor Necrosis Factor-α (TNF-α) and NF-κB gene transcription in brain of LPS-exposed mice were dramatically increased compared to those in the saline-injected mice (*P*=.01, *P*=.001). Treatment groups (KGF and CCF) were effective in attenuating LPS induced inflammatory signals by suppressive expression of IL-6, IL-1β, TNF-α and NF-κB.

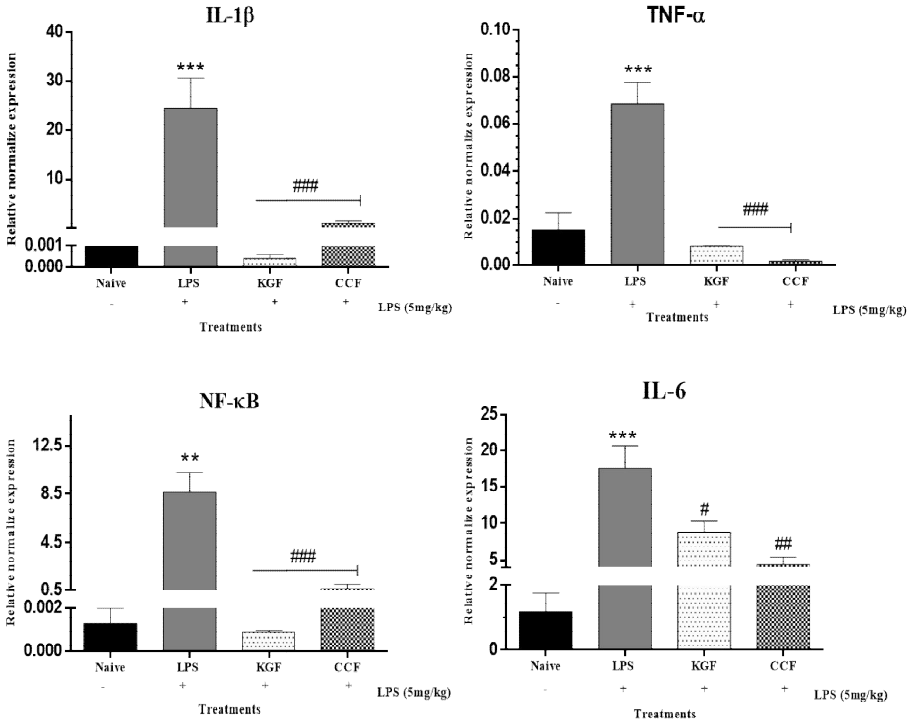

Fig. 2. KGF and CCF attenuated systemic LPS exposure-induced increases in pro-inflammatory mediators (cytokines (Interleukin-IL6; IL-1β and Tumor Necrosis Factor-TNF-α) and Nuclear factor κB, NF-κB) in the BALB/c mice brain

*Naive-Naive-saline solution treated mice; LPS- saline solution + LPS treated (5 mg/kg, i.p.) mice, KGF & CCF - K. grandifoliola and C. citratus polysaccharide fractions (100 mg/kg, p.o.) + LPS (5 mg/kg, i.p.) treated mice. The mRNA expression values (n=6) are given as mean ± SEM normalised to GAPDH levels in each sample. Y-axis values represent the number of mRNA copies relative to the number of GAPDH copies in the sample. #, ##, ### denote statistical significance at p<0.05, p<0.01 & p<0.001 in comparison to the LPS group. *, **, *** denote statistical significance at p<0.05, p<0.01 & p<0.001 in comparison to the naive group.Mean ± S.E.M = Mean values ± Standard error of means of six experiments*

4. DISCUSSION

One of the most important steps in evaluating the action of a compound on CNS is to perform neurotoxicity tests. Locomotor activity and muscle coordination are index of alertness and muscle relaxation. They measure the excitability threshold of the CNS and a decrease in motor activity and muscle relaxation is an indication of CNS depressant properties [21-22]. *K. grandifoliola* and *C. citratus* polysaccharides orally administered at 100 mg/kg did not cause a CNS depressant effect in BALB/c mice in actophotometer and on Rota rod bar as compared to naive group. In this study, systemic injection of LPS (5 mg/kg, *i.p*.) induces firstly hyperalgesia in BALB/c. The Hot plate test is an appropriate method to assess the effect of a centrally acting pain analgesic, while Tail-flick test is based on an acute spinal mediation reflex with harmful thermal stimuli [24]. It has been described that tail flick, licking or jumping occur when temperature at the level of nociceptors in the skin reach a critical value. If there was any damage in the peripheral nerves the latency of jumping or flicking would increase [8]. Many traditional drugs used to treat inflammatory diseases have shown a high ability to tolerance of stress in these two nociception models [25]. Thus, it was clear that KGF and CCF polysaccharides were analgesic and neuroprotective as indicated by their significant increase in % MPE higher or similar to animals of naive group. Their effects could result from inhibition of neuronal pain transmission in the presence of LPS [2].

Finally, the results of this study confirm that an injection (5 mg/kg , *i.p*) of LPS, mimics a systemic bacterial type infection as often occurs in mice and causes lasting acute neuroinflammatory changes through overexpression of four inflammatory mediators (IL-6, IL-1β, TNF-α and NF-κB) in brain. Similar results were obtained by [26], who showed that systemic LPS administration (2 mg/kg) resulted in a reduction of pain response latency in the tail flick test of neonatal rats and an increase of the levels of glial cells activation, pro-inflammatory cytokines IL-1β, cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in the spinal cord after 24 h. Although LPS-induced neuro-inflammation has been crucially associated with microglia activation astrocytes [27,28] play an important role in maintaining cerebral homeostasis and in protecting surrounding neurons from damage caused by infectious agents [2,6,29]. The mechanism of this prolonged CNS dysfunction involves the release of pro-inflammatory cytokines and partially activation of the NF-κB factor in astrocytes [30]. The mechanism by which cytokines and other pro-inflammatory mediators affect the brain and CNS functions, remains largely unexplored. According to [5] after systemic injection of LPS, TNF-expression in serum precedes the upward regulation of IL-1 suggesting that activation of the innate immune system and release of pro-inflammatory cytokines in systemic circulation negatively affect the functions of the CNS. TNF has already been reported as an early marker of mediation of cognitive decline after peripheral lesion contributing to the opening of the Hemato-Encephalic Barrier during systemic inflammation and neurodegeneration [31]. The role of IL-1β has been implicated in the modulation of pain sensitivity and mediating the hyperalgesia produced by LPS-induced inflammation [31]. The results obtained would suggest that pre-treatment of mice by FKG and HKG, provides protection against systemic LPS exposure-induced enhanced pain sensitivity and that the protective effects may be associated with is ability to attenuate transcription of TNF-α, IL-1β and IL-6 cytokines genes, depending on inhibition of NF-κB signalling pathway.

5. CONCLUSION

In summary, the current study demonstrated that systemic LPS exposure leads to brain inflammation and the enhancement of pain sensitivity (hyperalgesia) in BALB/c mice. These compromises are attenuated by *Khaya grandifoliola* and *Cymbopogon citratus* polysaccharide fractions, and the preventive effects may be associated with their ability to attenuate LPS-induced astrocyte activation and astrocyte-related pro-inflammatory cytokines as demonstrated earlier [18-19]. However further studies are required for determination of effective dose and mechanism of action associated in order to develop a drug against neuroinflammation- related diseases.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. Animals were handled in strict compliance with the Institutional Animal Ethical Committee Regulations (Approbation N° BITS-

HYD/IAEC/2016/21) for the care and use of laboratory animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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