Neuroprotective Effects of Ganoderma curtisii Polysaccharides After Kainic Acid-Seizure Induced

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ABSTRACT
Background: Epilepsy is one of the major neurological disorders affecting world population. Although, some Ganoderma species have shown neuroprotective activities, the effects of polysaccharides isolated from Ganoderma curtisii on epileptic seizures have not been reported. Objective: The aims of the present study were to determine whether treatment with a polysaccharide fraction (GCPS-2) from a Mexican Ganoderma curtisii strain can reduce seizures, and the increases in the levels of apoptotic molecules and inflammatory cytokines in kainic acid-induced seizure mouse model. Materials and Methods: Rats were separated in groups: Control group received 2.5% Tween 20 solution; GCPS-2 groups were administered GCPS-2 (10, 40, or 80 mg/kg); KA group received KA 10 mg/kg; GCPS-2+KA received GCPS-2 and 30 min later KA. Pathological changes in neuronal morphology, expression of B-cell lymphoma-2, and pro-inflammatory cytokines (interleukin1-β and tumor necrosis factor-α) in the rat hippocampus and cortex were determined by immunohistochemistry. Results: Ganoderma curtisii soluble polysaccharides (GCPS-2) inhibited convulsions in rats. Moreover, treatment with GCPS-2 reduced the increased levels of apoptotic signaling molecules (Bcl-2) and proinflammatory mediators (in the kainic acid-treated hippocampus and cortex). Conclusion: Ganoderma curtisii soluble polysaccharides have a neuroprotective potential against epilepsy, partially through its ability to inhibit neurotoxic events in the in vivo hippocampus and cortex. Key words: Ganoderma curtisii, β-glucan, Neuroprotective, Anticonvulsant, Anti-Inflammatory.

INTRODUCTION
Epilepsy is one of the major neurological disorders affecting approximately 1% of world population. Currently available antiepileptic drugs (AED) are associated with side effects, does not affect the underlying disorder, and about a third of patients are refractory to pharmacotherapies. A series of biophysiological and pathological reactions following seizures contribute to subsequent neuronal cell death. Apoptosis has been found as a way of cell death in pathological responses post-seizures. Determining the underlying mechanisms and developing potential therapeutic strategies for epileptic seizures are important research areas. The development of effective AED has been unaffected by the limited knowledge of the pathogenesis of this neurological disorder.1

Kainic acid was one of the first compounds used to study temporal lobe epilepsy (TLE). The administration of this L-glutamate analog causes neuronal depolarization and seizures, preferentially targeting the hippocampus. Injected rodents with kainic acid show recurrent seizures of variable frequency and hippocampus-restricted injuries. Neurodegeneration caused by injection of KA has been widely used in experiments to investigate mechanisms of excitotoxicity and possible pharmacological intervention against the seizure events. KA preferentially damages the pyramidal neurons of the CA3 area of the hippocampus, surviving neurons in the CA1 area of the hippocampus became hyperexcitable.2 Seizures are associated with some events such as inflammation, oxidative stress, neurotransmitter release, astrocyte proliferation, and microglia activation. Neuroinflammation plays a critical role in the pathogenesis of some neurodegenerative diseases. Although microglial activation is beneficial because it tends to diminish further injury and contributes to the repair of damaged tissues. Chronic activation of microglial cells endangers neuronal survival through the release of various proinflammatory molecules and neurotoxins such as nitric oxide (NO), proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α). Therefore, attenuation of microglial activation is considered a valuable strategy for the prevention and treatment of neurodegenerative diseases.3 Polysaccharides isolated from Ganoderma lucidum have been reported as neuroprotective agents against ischemia, inflammation, and neuroapoptosis.4 Ganoderma curtisii is a mushroom with few reported scientific studies, but there is not reports about the isolation of polysaccharides from G. curtisii nor the biological activity of these polysaccharides. Therefore, this study aimed to assess the anticonvulsant and neuroprotective effect of
**Ganoderma curtissi** poly saccharides in rats. Seizures were induced by intraperitoneal injection of kainic acid-seizure. Furthermore, whether the neuroprotective effects of GCPS-2 are associated with neuronal apoptosis and/or anti-inflammatory effects was investigated.

**MATERIALS AND METHODS**

**Ganoderma curtissi** strain

Ganoderma curtissi fruiting bodies were collected from a forest in the state of Morelos, Estados Unidos Mexicanos in August 2010. Identification of the mushroom was performed by M. C. Elizur Montiel and Dr. Isaac Tello, Laboratorio de Micología, Centro de Investigaciones Biológicas (CIB), Universidad Autónoma del Estado de Morelos (UAEM), Estados Unidos Mexicanos, and a voucher of the specimen (COBIOCh-UAEMOR-CF-G01) is deposited in the CIB Herbarium, UAEM.

**Phytochemical screening of GCPS-2**

Preliminary phytochemical screening of GCPS-2 was performed for the detection of chemical constituents: alkaloids, steroids, terpenoids, flavonoids, tannins, phenolics, saponins, carbohydrates, proteins and amino acids.

After the positive result for carbohydrates, the sugar composition of GCPS-2 (40 mg) was determined by acidic hydrolysis with 0.5 M trifluoroacetic acid for 5 h. After this time, the aqueous phase from the acid hydrolysis reaction was neutralized with Na₂CO₃ solution and lyophilized to give a white solid. Furthermore, the residue was dissolved in CH₃CN and analyzed by the HPLC system fitted with a NH₂ column. The sample was lyophilized and then stored at 4°C overnight. The precipitated and culture medium was centrifuged and filtered. The culture medium was bioalyzed yielding a dark brownish colored semi-solid mass (GCPS-2).

**Drugs administration**

In order to investigate the effects of GCPS-2 on preventing epileptic seizures, the drug was freshly prepared in 2.5% Tween 20 solution. Experiments were performed as following: GCPS-2 (10, 40, or 80 mg/kg) or kainic acid (KA, 10 mg/kg) were dissolved in 2.5% Tween 20 solution; and GCPS-2 or KA were administered i.p. to animals. Rats were separated in groups (5 rats per group): Control group received 2.5% Tween 20 solution; GCPS-2 groups were administered GCPS-2 (10, 40, or 80 mg/kg); KA group received KA 10 mg/kg; GCPS-2+KA received GCPS-2 and 30 min later KA.

**Monitoring of KA-induced seizures**

Animals of each group after KA treatment, were placed separately into transparent plexiglas cages (25 x 15 x 10 cm) and monitored for up 60 min in order to evaluate the time of seizure onset. The severity of seizures was classified into five stages: stage 1, characterized by facial movements; stage 2, characterized by head nodding and myoclonic twitching; stage 3, characterized by forelimb clonus with lordotic posture; stage 4, characterized by forelimb clonic seizures with reared posture, stage 5, characterized by tonic-clonic seizures without postural control; and stage 6, characterized by death of rats. In the present study, rats exhibiting seizures with at least stage 3 severity were considered as positive for seizure onset.

**Nissl staining of rat hippocampus and cortex**

Brain sections from sacrificed animals were exposed to the Nissl stain for the assessment of neuronal cell loss at hippocampus and cortex, as previously described. Two days after KA treatment animals were deeply anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and then transcardially perfused with cold saline followed by 4% paraformaldehyde in PBS (0.1 mol/L; pH 7.4). Brains were removed, washed in PBS, cryoprotected with 30% sucrose in PBS, and frozen in powdered dry ice. Coronal sections (6 μm) were cut at the level of the dorsal hippocampus (820 HistostatTM microtome). Every fourth sections were mounted on slides, dehydrated and rehydrated in graded ethanol and xylenes, respectively and then incubated in 1% cresyl violet for 30 seconds. Sections were then decolorized in acid acid, dehydrated, and coverslipped. Sections were observed with a binocular microscope (Primo Star, Carl Zeiss).

**Immunohistochemistry staining**

Brain sections were prepared for immunohistochemistry staining following a procedure optimized in our laboratory. In brief, after two consecutive PBS washes, sections were incubated overnight at 4°C in PBS-BSA containing the primary antibodies: B-cell lymphoma-2 (Bcl-2, 1:50); glial fibrillary acidic protein (GFAP, 1:100 dilution), IL-1β (1:100 dilution), mouse anti-Hsp60 monoclonal antibody (1:1000 dilution).
dilution), mouse anti-Hsp70 monoclonal primary antibody (1:1000 dilution), and mouse anti-neuronal nuclei (NeuN, 1:200 dilution). The sections were subsequently washed and incubated with biotin-conjugated secondary antibodies, followed by an avidin biotin complex kit. The stained sections were examined under a microscope (Primo Star, Carl Zeiss).

Counting of hippocampal CA1, CA2, and CA3 neurons
The number of hippocampal CA1, CA2, or CA3 neurons was quantified using alternate sections prepared from the coronal brain slices of animals. Neurons with visible nuclei were counted using a light microscope according a procedure optimized in our laboratory (Primo Star, Carl Zeiss).

Statistical analysis
Statistically comparisons for significance among the different groups of animals were performed by ANOVA and Tukey test. Statistical comparisons for significance between cells were performed via Student’s t-test. The differences were considered statistically significant when P < 0.05. Data are expressed as the arithmetic mean ± standard error.

RESULTS
Phytochemical screening of GCPS-2
The chemical tests used for preliminary phytochemical analysis indicated that no triterpene, flavonoid, saponin or protein compounds were detected in this fraction. The only positive test was for saccharides. The yield of the soluble polysaccharide fraction GCPS-2 was 3 g/L Sugar compositional analysis of GCPS-2 was performed by acid hydrolysis of the polysaccharide and HPLC analysis of the hydrolyzed, indicated that polysaccharide was composed by glucose (t_R = 12.2 min) units.

In the 1H NMR spectrum of GCPS-2 (Figure 1), a weak anomic signal was identified at 5.18 ppm with a coupling constant (\( J_{H1-H2} \)) of 3.1 Hz. Additionally, another weak anomic signal was observed at 5.22 ppm with a similar coupling constant value. These values of \( J_{H1-H2} \) indicate an alpha (α) configuration for glucose units. There were also two higher intense anomic signals at 4.43 and 4.62 ppm with a \( J_{H1-H2} = 9.1 \) Hz corresponding to a beta (β) configuration. The proportions of the integrated peak areas for these signals were 1:9:9 respectively, indicating that β-glucan was the most abundant compound in GCPS-2. The \(^{13}\)C NMR spectrum of GCPS-2 (Figure 2) show signals at 98.2 and 95.9 ppm corresponding to a β configuration, and signal at 94.5 ppm corresponding to an α configuration. The branching points at C-6 were shown by the -CH_2-O- signals at 66.7 ppm in the spectrum, compared to the unsubstituted -CH_2-OH signals at 63 ppm. In addition, MALDITOF mass spectrometry indicated signals in the range 40 000 - 45 000 Da for GCPS-2.

GCPS-2 pretreatment inhibits seizures
The KA excitotoxicity was established to induce seizures; behavioral changes and neuro-histochemical features similar to those found in patients with TLE. Rats administered with KA (10 mg/Kg) showed the stages 1 to 5 of seizure severity including the stage 6 (death of one animal). The first stage of neurotoxicity in rats occurred at 90 min after KA administration. To determine the effects of GCPS-2 on KA-induced seizures, various doses of GCPS-2 (10, 20, 40, 80 mg/Kg) were i.p. administered 30 minutes before the KA injection. The effect of GCPS-2 in inhibiting seizures was not dose-dependent, at all doses rats only showed behavior of stage 1. Rats treated with GCPS-2 at various doses (10, 20, 40, 80 mg/kg) did not develop any stage of seizure severity.

Neuroprotective effects of GCPS-2
Hippocampal and cortex sections of rats from all groups were stained with hematoxylin and eosin (H&E) to detect degenerated neurons (Figure 3). We can observe that in control group there was no evidence of neuronal damage. A preservation of cortex neurons was found in rats treated with GCPS-2 (10 mg/kg). The majority of neurons in cortex sections in the group treated with KA (10 mg/Kg), appeared shrunken with eosinophilic cytoplasm, triangulated pyknotic nuclei, and edema (Figure 3C). In contrast, rats pre-treated with GCPS-2 (10 mg/Kg) did not show a significant decrease in the number of neurons after KA injection (Figure 3D). In the graph (E), we can observe that there are significant differences (p = 0.0058) in cell damage, between

Figure 1: 1H NMR spectrum of GCPS-2 (400 MHz, D_2O).
Effect of GCPS-2 on NeuN

NeuN labeling was used to encompass neuronal cell losses by staining for detecting neurons in the process of degeneration. After KA injection, the numbers of NeuN-positive cells in cortex sections were significantly decreased. Also, a significant loss of cells, interstitial edema and cellular damage was observed in the cortex. Group pre-treated with GCPS-2 showed a similar expression of NeuN-positive neurons to control group (Figure 7). Group that received GCPS-2, showed some cells in the process of neurodegeneration (B).

Effect of GCPS-2 on HSP70 and HSP60 expression

In order to determine whether GCPS-2 protects against kainic acid neurotoxicity the HSP70 or HSP60 expression levels in the cortex of different experimental rat groups were determined. As shown in Figure 5, HSP70 expression levels were significantly higher in the KA group compared with the control group. Administration of GCPS-2 (B) did not induce an increase in the expression of HSP70, being the levels similar to that of control group (A). Kainic acid induced an elevated expression of HSP70, meanwhile in group with pretreatment of GCPS-2, the HSP70 immunoreactivity decreased in comparison with KA group as shown in (D). The expression of HSP60 was determined also by immunostaining (Figure 6). In KA group, HSP60 immunoreactivity was increased in the hippocampus and cortex regions (C). In animals pretreated with GCPS-2 and a posterior KA administration, HSP60 immunoreactivity decreased in comparison with KA group (D) (Figure 6). Animals treated with GCPS-2 (B), showed an expression of HSP60 similar to that of control group (A).

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GCPS-2 attenuates KA-induced apoptotic molecules expression

To examine whether GCPS-2 pretreatment inhibits KA-induced apoptosis, the expression levels of Bcl-2 an apoptotic molecule was determined in samples of hippocampus and cortex brain tissues. Basal expression of Bcl-2 was detected in hippocampus and cortex samples from the control group. Meanwhile, animals treated with GCPS-2 showed a level of Bcl-2 expression similar to that of control group. A great Bcl-2 positive staining was visible in hippocampus and cortex sections of rats subjected to KA when compared with control group, demonstrating that KA treatment significantly enhanced the expression of Bcl-2. Furthermore, the increased levels of Bcl-2 in the KA-treated hippocampus and cortex sections was significantly attenuated by pretreated with GCPS-2, compared with KA group (Figure 5).

GCPS-2 downregulates pro-inflammatory mediators in KA-treated rats

To determine whether GCPS-2 affects the expression of inflammatory mediators such as IL-1β and TNF-α, which may be produced by
activated microglia, in the hippocampus and cortex sections were stained. The results obtained (Figure 6) demonstrate that KA treatment resulted in microglial activation and an increase in IL-1β and TNF-α in hippocampus and cortex. GCPS-2 pretreatment inhibited these increases in inflammatory mediators. These results suggest that GCPS-2 protects hippocampal and cortex neurons by inhibiting the production of pro-inflammatory mediators.

**DISCUSSION/CONCLUSION**

Studies on Ganoderma species have been performed mainly on Asian and European strains, but few studies have considered Mexican strains. And besides, *Ganoderma curtisii* is a mushroom that has been few studied. This is the first report of isolation of polysaccharides from *Ganoderma curtisii*. Aguilar et al. reported the polyphenol content, and Yang et al., isolated lanostane triterpenoids from fruiting bodies of *G. curtisii*, but they did not isolate polysaccharides. 10,11 Several reports have shown that β-glucans have been isolated from fruiting bodies of mushrooms, but few reports mention the isolation of β-glucan from submerged culture of mycelium. 12,13 This is the first report of β-glucan isolation from submerged cultivation of *G. curtisii*.

Although new antiepileptic drugs have shown advantages as anticonvulsants, their efficiency is limited. The search for new molecules with neuroprotective activities has been performed on plants and mushrooms. There are several mushrooms which have been reported as an interesting source of metabolites, with the ability to promote cure on brain affections. Polysaccharides are one type of metabolites that have shown neuroprotective properties against brain injury induced by neurotoxins and stroke. The beneficial neuroprotective effects of polysaccharides consider the capacity to suppress neuroinflammation and excitotoxicity with minimal side effects. There are few reports...
describing anticonvulsant activity of polysaccharides isolated from Ganoderma species.\textsuperscript{14,15} The anticonvulsant activity induced by GCPS-2 is similar to that reported by us with an oligosaccharide fraction from a Ganoderma lucidum strain collected in a Mexican forest.\textsuperscript{16} These results suggest that GCPS-2 is a natural compound with potential anticonvulsant activity.

Kainic acid administered to rats induced seizures and caused hippocampal and cortex neuronal death. The neuronal death may be due to apoptosis triggered by the activation of several transduction factors as caspase-3, Bcl-2, and Bax,\textsuperscript{17} suggesting that controlling apoptosis may be an important protection of hippocampus and cortex following epileptic seizures. Pretreatment with GCPS-2 protected the hippocampus and cortex neurons against KA-induced excitotoxicity. And besides, GCPS-2 prevented the increase of Bcl-2 levels following the KA injection. These results suggest that GCPS-2 pretreatment attenuated the apoptotic signal induced by KA treatment, resulting in protection from neuronal degeneration. GCPS-2 pretreatment also protected the hippocampal and cortex neurons against neurotoxicity.

As a default response to injuries caused by excitotoxicity and a variety of neurodegenerative diseases (including epilepsy), cells induce a variety of proteins among them heat shock proteins that confers cellular protection against insults.\textsuperscript{18} In the present study, the expression levels of Hsp60 or Hsp70 expression increased by the administration of KA. These results and the Bcl-2 expression suggest that neuronal apoptosis could be implicated in the kainic acid-seizure induction. KA may initiate a neuronal apoptosis cascade that contributes to substantial neuronal damage, convulsions and behavioral impairment. The pretreatment with GCPS-2 reduced the expression of HSP60 or HSP70 after the administration of KA, showing the neuroprotective effects of GCPS-2. The results of the present study suggested that anti-apoptotic proteins may serve as potential therapeutic targets for the protection of damaged brain in cases of kainic acid-seizure induction. Previous studies have shown that the administration of Ganoderma lucidum extracts (containing polysaccharides and ergosterol) exert neuroprotective effects by the production of microglia-derived proinflammatory and cytotoxic factors. The results obtained by us show for first time that G. curtisii mycelium produces polysaccharide with neuroprotective activity on rats subjected to kainic acid-seizure induction, and that an apoptotic mechanism may be involved in the neuroprotective effect of GCPS-2.

Neurotoxicity events in the brain following epileptic seizures implies increase in apoptotic signaling molecules and in the levels of inflammatory mediators (IL-1β, TNF-α, etc). The control of these...
inflammatory pathways may be one the many potential strategies for the prevention of seizures. The results of this study demonstrate that pretreatment with GCPS-2 inhibit microglial activation in KA-treated hippocampus and cortex of rats, resulting in a decrease of levels of IL-1β and TNF-α. These results suggest that the anti-apoptotic and anti-inflammatory effects of GCPS-2 are associated with the protection of hippocampal and cortex neurons from KA-induced neurotoxicity.

The present study showed the neuroprotective effects of GCPS-2 isolated from a *Ganoderma curtisii* strain collected in Mexico, against KA-induced seizures in vivo. Our results demonstrate that GCPS-2 inhibit convulsions in KA-seizure induced model. In addition, we determined that the neuroprotective properties of GCPS-2 against KA-induced excitotoxicity is mediated by the inhibition of microglial activation and apoptotic signaling molecules in hippocampus and cortex. These results suggest that GCPS-2 is a potential natural compound for the prevention and treatment of epileptic seizure.

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**REFERENCES**

GRAPHICAL ABSTRACT

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