



Evaluation of the effects of *Astragalus mongolicus* Bunge saponin extract on central nervous system functions

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ABSTRACT

Ethnopharmacological relevance: In traditional medicine, *Astragalus mongolicus* (AM) has been used for the treatment of general weakness, chronic illness, and to increase overall vitality.

Aim of the study: The present study investigated possible effects of the saponin fraction of AM on the central nervous system. Moreover, its effects on locomotor activity, anxiety, and hippocampal morphology were studied.

Material and methods: AM extract was tested for its effects on locomotor activity using the Moti-Test, for situational anxiety in the elevated plus maze, and for anticonvulsant activity against acute pentylenetetrazole (PTZ)-induced seizures and in the PTZ kindling model.

Results: It was shown that AM (50, 100, 200 mg/kg) did not interfere with locomotor activity and situational anxiety as measured in the elevated plus maze. In these doses, AM significantly suppressed pentylenetetrazole (PTZ)-induced seizures ($p < 0.05$). Its anticonvulsant efficacy was also evident against repeated PTZ seizures ($p < 0.05$). This suggests potential therapeutic usefulness. After subchronic application, the number of cells in hippocampal CA1 was reduced, whilst the cell number in CA3 and hilus remained unaffected.

Conclusions: Doses of AM extract which did not interfere with locomotor activity and situational anxiety appear to be useful in the treatment of convulsive disorders. The mechanisms underlying this effect on hippocampal morphology are not yet understood.

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1. Introduction

The main ingredients of *Astragalus membranaceus* var. *mongolicus* (synonym *A. mongolicus*, AM) are polysaccharides (Kitagawa et al., 1983a), saponins, and flavonoids (Kitagawa et al., 1983b,c,d; Aldarmaa et al., 2010). The root of the *Astragalus* plant is typically used in soups, teas, extracts, or capsules. There is no recent clinical evidence to guide dosages of *Astragalus* products. However, typical recommendations are 2–6 g of the powdered root (Monograph, 2003). In traditional medicine, AM has been used for the treatment of general weakness, chronic illness, and to increase overall vitality. Different peripheral effects such as improved sensitivity to insulin (Lin et al., 2000), immune modulation, antiviral activity, antineoplastic activity, and enhancement of cardiovascular functions have been described (Monograph, 2003). The protection of cardiovascular function might be explained in terms of protection against membrane lipid peroxidation (Chen et al., 1995; Wang et

al., 1996; Toda and Shirataki, 1999). As well as peripheral effects, a variety of central effects have been described. Behavioural studies demonstrated antiamnestic effects (Molodavkin et al., 2000). This was confirmed by subsequent experiments demonstrating that AM extract reversed Aβ(25–35)-induced memory loss and prevented the loss of axons and synapses in the cerebral cortex and hippocampus of mice (Kitagawa et al., 1983a). An investigation of conflict situations in rats showed that AM had antidepressant and anxiolytic effects (Molodavkin et al., 1998, 2000). It is noteworthy that both effects occurred in the absence of sedation and muscle relaxation (Molodavkin et al., 1998) and tranquilizer-typical changes in the electroencephalogram (Molodavkin et al., 2000). More recently, it was reported that AM extract was effective against acute pentylenetetrazole (PTZ)-induced seizures in mice, but not against electrically evoked seizures in rats (Aldarmaa et al., 2010). AM extract was also found to reduce oxidative stress following PTZ convulsions in mice (Aldarmaa et al., 2010). It was concluded that the extract could represent a useful additive for antiepileptic therapy in preventing the development of cognitive impairment. To further characterise the potential of AM extract, the effects on locomotor activity, on elevated plus

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maze behaviour, and on PTZ-induced seizures were tested in rats.

2. Materials and methods

Ethical approval was sought according to the requirements of the National Act on the Use of Experimental Animals (Germany).

2.1. Animals

Experiments were carried out with male Shoe:Wist(Shoe) rats from our own breeding colony aged 8 weeks at the beginning of the behavioural experiments. The rats were kept under controlled laboratory conditions (light regime of 12 h light/12 h dark, light on at 6:00 a.m., temperature $20 \pm 2^\circ\text{C}$, air humidity 55–60%). They had free access to commercial rat pellets (ssniff R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. After weaning, the rats were housed in groups of five per cage.

2.2. Behavioural test

Behavioural tests were performed in the light period between 8.00 a.m. and 2.00 p.m. All substances were solved in 0.9% NaCl solution (sal). AM was given orally by gavage, and pentylenetetrazol (PTZ; Roth, Karlsruhe, Germany) was ip injected. For control 0.9% sal was given. The application volume was 10 ml/kg body weight. All solutions were freshly prepared.

2.3. Preparation and chemical characterisation of AM extract

2.3.1. Preparation of extract

The root of *Astragalus mongolicus* (AM) was collected from the Gachuur region of Ulaanbaatar in October 2007. It was identified with the standard sample preserved in the Department of Botany, Institute of Biology, Mongolian Academy of Sciences. Astragalus saponins were extracted according to the method by Ma et al. (2002). The dried roots of AM (0.5 kg) were extracted with ethanol at 40°C at ratio of 1 g:5 ml for 7 days, and the filtrate was evaporated under vacuum at less than 45°C to give a final yield of 20 g. Twenty gram crude extract was extracted with chloroform and ethyl acetate. Chloroform and ethyl acetate extracts were discarded. The residue was dissolved in methanol (200 ml), filtered and concentrated to 65 ml. 1-Butanol was added to the residue from methanol concentrated phase separation to obtain total saponins. The crude extract contained 10% (w/w) of total saponins, mixture acetylastragaloside I–VII, and acetylastcaguloside I.

The precipitate was filtered, dried at 105°C and stored in a refrigerator until further use. During the experiments, the prepared total saponins of AM were dissolved in deionized water and used in all experiments.

2.3.2. Standardization of extract

Extract 5 g of AM powdered root for 10 min with 100 ml of a mixture ethanol water (57:43). Filter and evaporate filtrate under vacuum at 45°C . Remove lipids from the concentrate by treating 10 ml of a mixture of ethyl acetate water (1:1). Remove the upper phase and extract the remaining lower phase with two 5 ml ethyl acetate. Discard ethyl acetate. Extract aqueous phase three times with 5 ml 1-butanol. The combined extracts are washed with three times 2 ml portions of water. 1-Butanol is removed under vacuum and the residue is dissolved in 1 ml methanol. Standard: 1 mg of astragaloside IV in 1 ml methanol. Mobile phase: chloroform:methanol:water 18:8:1 in 366 nm.

2.4. Acute effects of AM

In the first experiment, the acute effectiveness of AM (50, 100, and 200 mg/kg) after oral administration was evaluated in a test battery.

2.4.1. Activity

A computerised system (Moti-Test, TSE, Bad Homburg, Germany) was used to measure activity 40 min after oral substance administration. The system consisted of 4 identical boxes ($46\text{ cm} \times 46\text{ cm} \times 50\text{ cm}$) equipped with 15 infrared cells on each of the X-Y (plane)-Z (height) levels to measure horizontal and vertical activity. The illumination level in the sound-reduced testing room was 30 lx. The boxes were cleaned and wiped prior to the first test and after each test. After a pretreatment time of 40 min, the animals were exposed to the system for 20 min. Activity time (i.e. time spent in horizontal + time spent in vertical activity) was measured at 5 min intervals.

2.4.2. Elevated plus maze

Anxiety was measured in the elevated plus maze under low-light conditions (30 lx) immediately after the Moti-Test. The maze was made of black polyvinyl chloride and had two open and two closed arms ($50\text{ cm} \times 10\text{ cm} \times 40\text{ cm}$) mounted 50 cm above the floor. The floor of the arms was smooth.

A rat was placed in the central platform of the apparatus facing a closed arm. A camera on the ceiling of the test room was used to score and tape the animals' behaviour from an adjacent room for a period of 7 min. The number of entries into open arms, time spent on open arms and time spent on closed arms were measured. The maze was cleaned after each trial.

2.4.3. Acute PTZ seizures

Following the elevated plus maze, i.e. 70 min after oral administration of AM, 40 mg/kg PTZ ip was given. Resultant seizures were scored as described below for a period of 20 min.

For comparison, separate groups of rats were dosed with 0.5 or 2.5 mg/kg diazepam (DZP, Faustan®, AWD Dresden, Germany) at corresponding times.

2.5. Subchronic application

In the second experiment, AM effects in a subchronic experiment were investigated. The substance was given 60 min prior to PTZ or sal in the course of kindling induction. In the acute experiment it was found that the anticonvulsant effects of the three doses (50, 100, and 200 mg/kg) tested did not differ. Therefore, the 50 mg/kg dose was tested in the subchronic experiment alone.

In separate groups of rats, the ED16 related to clonic-tonic seizures was established (37.5 mg/kg). Injections were given on Monday, Wednesday, and Friday. After each injection, the convulsive behaviour was observed for 20 min. The resultant seizures were classified as follows:

- stage 0: no response
- stage 1: ear and facial twitching
- stage 2: myoclonic jerks without rearing
- stage 3: myoclonic jerks, rearing
- stage 4: turning over into side position, clonic-tonic seizures
- stage 5: turning over into back position, generalised clonic-tonic seizures

In total, rats received 13 convulsant injections. Control animals received the same number of saline injections.

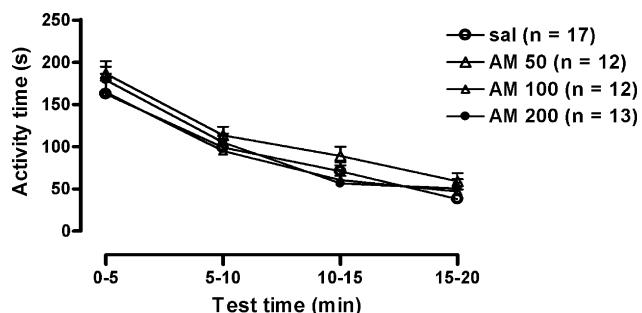


Fig. 1. Activity (time spent in vertical + time spent in horizontal activity) in rats orally treated with either saline, 50 mg/kg, 100 mg/kg, or 200 mg/kg *Astragalus mongholicus* extract (AM). N = number of animals tested. Mean \pm SEM.

2.6. Histology

One week after completion of the seizure experiment, the animals were anaesthetised with chloral hydrate, and were transcardially perfused with 200 ml of 0.9% saline followed by 200 ml 8% formaldehyde. The brains were removed and placed into fixative solution for 24 h. After dehydration, the brains were embedded in paraffin. In order to perform a histological investigation, coronal paraffin sections 10 μ m thick were cut in the area of the nucleus habenulae. The sections were stained with toluidine blue.

Cells in hippocampal CA1, CA3 and hilus were counted in squares of 245 μ m \times 245 μ m using a counting net. There was an average of ten fields in the left and right hippocampus per animal. The histological analysis was performed blindly.

2.7. Statistics

The data obtained were analysed using ANOVA followed by Bonferroni post hoc. To evaluate activity and seizure development, the repeated measure model was applied. Seizure stages were analysed using the Kruskal-Wallis H-Test followed by the Mann-Whitney U test. The basis of statistical decision was a significance level of 0.05. Data were expressed as Mean \pm standard error of the Mean (SEM). The calculations were carried out using SPSS+ software.

3. Results

3.1. Activity

AM was tested after oral application in doses of 50, 100, and 200 mg/kg. As shown in Fig. 1, there are no differences in activity between controls and AM treated animals ($F_3, 50 = 1.23, p = 0.309$). Activity declined in all experimental groups ($df = 3, F = 252.95, p < 0.001$) and there was no time \times group interaction ($df = 9, F = 0.973, p = 0.465$).

3.2. Elevated plus maze

After oral administration of AM, none of the parameters measured on the elevated plus maze was altered (open arms: $F_3, 53 = 1.335, p = 0.273$; % time spent in open arms $F_3, 53 = 0.491, p = 0.69$; arm changes $F_3, 53 = 1.335, p = 0.273$). This indicates that AM had no effect on situational anxiety (Fig. 2).

3.3. Acute PTZ seizures

As shown in Fig. 3, all doses (i.e. 50, 100, and 200 mg/kg) significantly reduced seizure severity (Kruskal-Wallis $df = 3, \chi^2 = 7.797$,

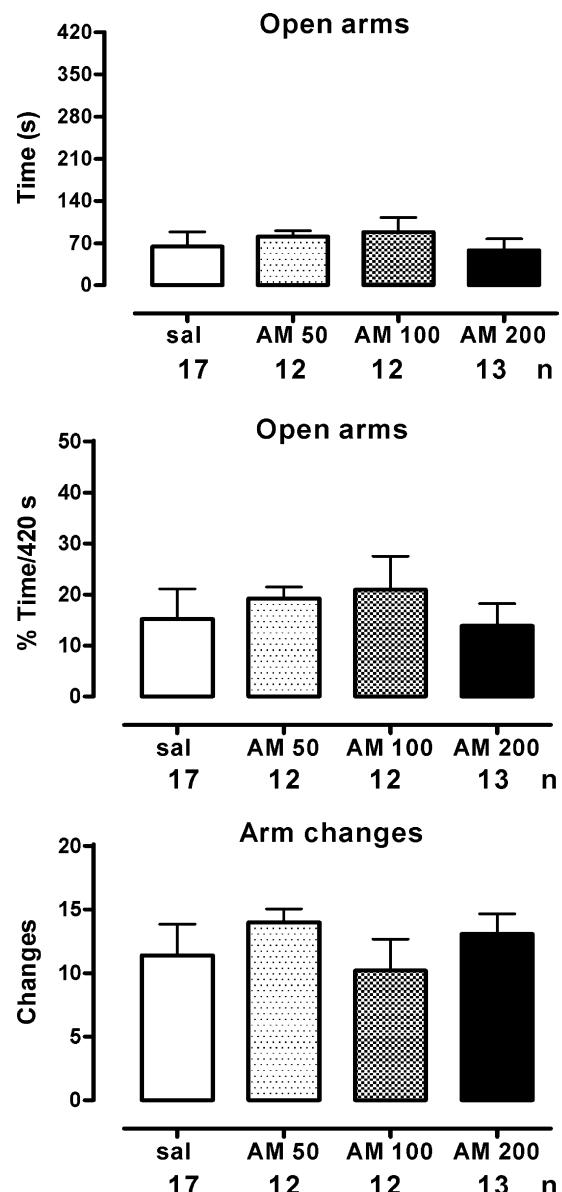


Fig. 2. Effect of *Astragalus mongholicus* extract (AM, 50 mg/kg, 100 mg/kg, 200 mg/kg) on behaviour on the elevated plus maze. Control animals were treated with saline (sal). N = number of animals tested. Mean \pm SEM.

$p = 0.05$, post hoc Mann-Whitney AM 50 U 9, 7 = 13, $p = 0.045$, AM 100 U 9, 9 = 17, $p = 0.034$, AM 200 U 9, 9 = 13.0, $p = 0.013$). Interestingly, the anticonvulsive effect of AM showed no dose-dependency. For comparison, the effect of DZP was shown (sal vs. 0.5 mg/kg U 17; 10 = 57, $p < 0.04$, sal vs. 2.5 mg/kg U 17, 12 = 50, $p < 0.05$).

3.4. Subchronic PTZ seizures

This was designed as a kindling experiment. However, in the main experiment the animals reacted with greater sensitivity. Instead of kindling, we investigated the AM effect in a subchronic seizure experiment. The course of seizure severity was parallel (day \times group $df = 12, F = 0.52, p = 0.9$), but AM-pretreated rats had lower seizure scores compared with respective controls ($F_1, 16 = 4.29, p = 0.05$), Fig. 4. This clearly indicates that repeated AM treatment did not alter the anticonvulsive effectiveness found in the acute experiment.

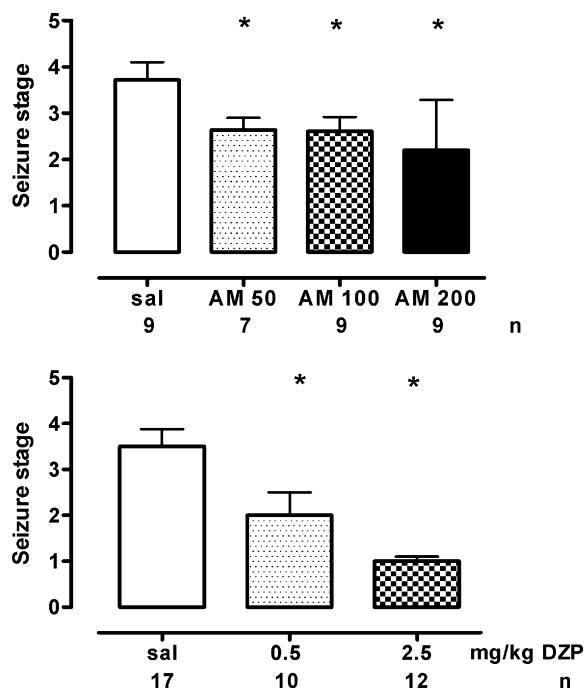


Fig. 3. Effect of *Astragalus mongolicus* extract (AM, 50 mg/kg, 100 mg/kg, 200 mg/kg) on acute seizures induced by 40 mg/kg pentylenetetrazole (PTZ). PTZ was given 70 min after oral AM administration. For comparison, separate groups of animals were treated with either 0.5 mg/kg or 2.5 mg/kg diazepam. sal = saline, DZP = diazepam. N = number of animals tested. Mean \pm SEM. * p < 0.05, U test.

3.5. Histology

As shown in Fig. 5, the number of CA1 neurones in CA1 was significantly reduced after AM treatment ($F_{1, 20} = 9.835, p = 0.006$), whereas the number of CA3 cells ($F_{1, 20} = 2.034, p = 0.172$) and cells in hilus ($F_{1, 20} = 2.061, p = 0.169$) remained unchanged. A similar effect was found after repeated seizures. Repeated seizures resulted in significant neurone loss in hippocampal CA1 cells ($F_{1, 20} = 5.84, p = 0.027$) but not in CA3 ($F_{1, 20} = 0.09, p = 0.768$) and in the hilus region ($F_{1, 20} = 1.753, p = 0.203$). There was no seizure \times AM treatment interaction ($p > 0.05$). Comparison of the cell number in the CA1 region resulted in a significant difference between the 4 experimental groups ($F_{3, 20} = 6.939, p = 0.003$). Post hoc analysis revealed a significantly lower cell number in the treated groups (AM/sal, sal/PTZ, AM/PTZ) compared with sal/sal ($p < 0.05$, Bonferroni test).

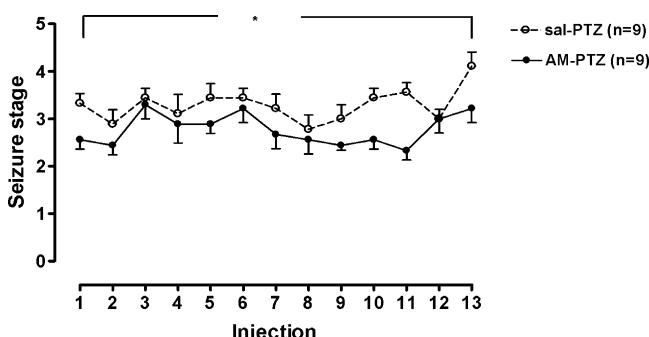


Fig. 4. Effect of *Astragalus mongolicus* extract (AM) on repeated seizures induced by 37.5 mg/kg pentylenetetrazole (PTZ). PTZ was given 60 min after oral AM administration. sal = saline. N = number of animals tested. Mean \pm SEM. p < 0.05.

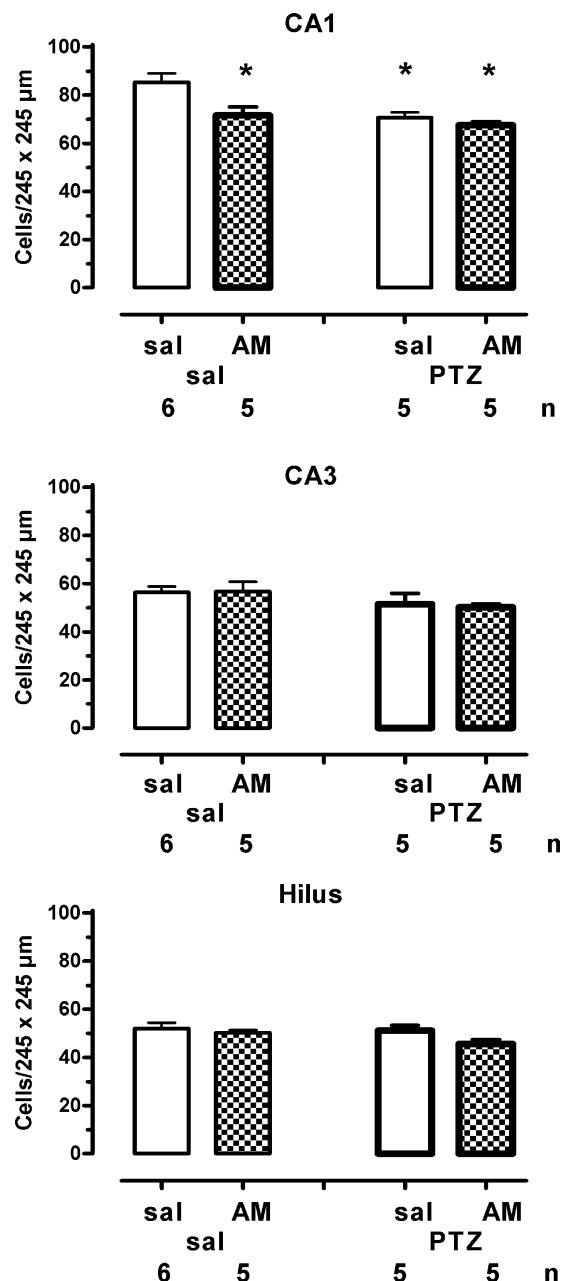


Fig. 5. Influence of *Astragalus mongolicus* extract (AM) on the number of neurones in the rat hippocampus. Number of neurones \pm SEM counted in a field of $245 \mu\text{m} \times 245 \mu\text{m}$ in control animals (sal/sal), AM controls (AM/sal), after repeated seizures (sal/PTZ) and PTZ-injected rats pretreated with AM (AM/PTZ). sal = saline, PTZ = pentylenetetrazole. N = number of animals tested. * p < 0.05 in comparison with sal/sal.

4. Discussion

AM extract has been used in traditional medicine for the treatment of general weakness, chronic illness, and to increase overall vitality (Lin et al., 2000). A recent report demonstrated effectiveness against acute seizures induced by the chemoconvulsant PTZ (Aldarmaa et al., 2010) and anxiolytic and antidepressant properties measured in a conflict test (Molodavkin et al., 2000). Assuming that the main AM effects were mediated via GABA/benzodiazepine receptors, interferences with locomotor activity and effects in other tests of anxiety could be expected. In the present experiments, different doses of AM extract showed effects neither on locomotor activity (Fig. 1) nor on anxiety measured on the elevated plus maze

(Fig. 2). This tallies well with earlier experiments showing that AM was not sedative (Molodavkin et al., 1998). The lack of effectiveness on plus maze behaviour does not necessarily contradict the anxiolytic/antidepressant effects found in the conflict situation, since both tests reflect different states of anxiety. The conflict test was based on punished behaviour, whereas the elevated plus maze used in our experiments is based on exploratory behaviour (File, 1992). Moreover, differences in the behaviour of rats are not only determined genetically, but also by preceding handling procedures (Schmitt and Hiemke, 1998).

Electrophysiological studies revealed that oral AM markedly modified the EEG. The spectral power of the τ rhythm decreased, whilst other frequency bands remained unchanged. The effect was detected in sensorimotor cortex and hippocampus and peaked 60–90 min after application (Molodavkin et al., 1998). For control, phenazepam was tested. This substance also decreased the power of the τ rhythm, but it increased the power in β and δ frequency bands (Molodavkin et al., 1998). A decrease in τ peak frequency is commonly used as a biomarker for anxiolytic effects (Bouwman et al., 2004). This biomarker not only holds for GABAergic anxiolytics but also for other non-GABAergic anxiolytics (McNaughton and Coop, 1991).

It was shown that for the benzodiazepines, there is a close correlation between an increase in β frequency spectral power and anticonvulsant activity (Mandema et al., 1991). In our experiments, AM significantly suppressed PTZ-induced seizures (Fig. 3). Even after long-term administration, this effect was evident (Fig. 4). It is well accepted that anti-PTZ efficacy reflects interaction with GABA/benzodiazepine receptors. Interestingly, AM extract did not interfere with the β band (Molodavkin et al., 1998). This suggests that anticonvulsant effects as found in mice (Aldarmaa et al., 2010) and in the present study (Figs. 3 and 4) are possibly unrelated to the GABA/benzodiazepine receptor. The mechanism underlying anticonvulsant effectiveness remained undisclosed.

AM was shown to reverse amyloid peptide A β (25–35)-induced memory loss and prevented the loss of axons and synapses in the cerebral cortex and hippocampus in mice (Tohda et al., 2006). This is in line with other reports on protection against oxidative damage, mitochondrial dysfunction (Aldarmaa et al., 2010), and radical scavenge (Gee et al., 2006). As a consequence of PTZ-kindling, the number of neurones in the hippocampal CA1 region was found to be reduced (Pohle et al., 1997; Becker et al., 1997). In order to study AM effects on neuronal survival after repeated seizures, cells in hippocampal CA1, CA3, and hilus were counted. As shown in Fig. 5, the cell number in CA3 and hilus did not differ between the 4 experimental groups. Surprisingly, in the CA1 region, sub-chronic administration of AM induced neuronal loss similar to that seen in the PTZ convulsion group. Seizures induce transient cerebral ischemia which causes an inhomogeneous pattern of cell death in the brain. Within the hippocampus, CA1 cells were found to be more susceptible, whereas CA3 neurones are more resistant to ischemic events (Benveniste et al., 1989; Diemer et al., 1992; Gee et al., 2006; Dos-Anjos et al., 2009). It was speculated that different calcium-dependent modulation of NMDA currents (Grishin et al., 2004) and energy deprivation differentially shifts the intracellular equilibrium between tyrosine kinase and phosphatase activities that modulate NMDA responses in CA1 and CA3 neurones (Gee et al., 2006). As it stands we do not yet have any information about the effect of AM on hippocampal cell energy metabolism.

The present study confirms saponin AM extract anticonvulsant activity in rats and, moreover it demonstrated the effectiveness of the extract in a developmental model of epilepsy. Subsequent experiment are designed to clarify as to what extent other fractions contribute to the effects found. Preliminary results indicate the polysaccharide fraction did not contribute to anticonvulsant efficacy.

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