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In vitro and *in vivo* anti-seizure activity of hydromethanolic extract and fractions of *Pterolobium stellatum*

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ABSTRACT

Ethnopharmacological relevance: In Ethiopia, the whole plant juice of *Pterolobium stellatum* is used to treat seizures and epilepsy.
Aim of the study: To investigate the antiseizure activity of hydromethanolic crude extract and fractions collected from leaves of *P. stellatum* using both *in vitro,* and *in vivo* seizure models in mice.
Materials and methods: Fresh leaves of *P. stellatum* were collected from Awash Melka, Addis Ababa, Ethiopia. An 80% crude methanol extract was further fractionated to produce petroleum ether, chloroform, butanol, and aqueous fractions. Anti-seizure activity of the crude extract and fractions (petroleum ether, chloroform, butanol, and water) were assessed at a concentration of 0.7 mg/ml using the *in vitro* 0 Mg^{2+} model of seizures in mouse brain slices prepared from 14- to 21-day-old C57BL/6 mice. The maximal electroshock seizure (MES) model and the pentylenetetrazol (PTZ) seizure model for seizures were performed on male BALB/c mice using 400 mg/kg and 800 mg/kg of crude 80% methanol extract, as well as the four fractions described above. Diazepam and phenytoin were used as positive controls for PTZ and MES test respectively.
Results: Addition of 0.7 mg/ml of crude 80% methanol extract of *P. stellatum* prevented the onset of SLEs in most brain slices in the 0 Mg^{2+} *in vitro* model of seizures, with similar efficacy to diazepam (3 μM). The same extract at 400 and 800 mg/kg was efficacious in reducing the hindlimb extension time in the MES model and delaying the onset of myoclonic convulsions in the PTZ model, although not to the same extent as phenytoin (10 mg/kg) or diazepam (5 mg/kg). The chloroform and water fractions of the crude extract also showed significant anti-seizure activity across all three models whilst the non-polar petroleum ether and butanol fractions did not. The UPLC-MS analysis indicated the presence of gallic acid, ellagic acid, kaempferol, myricitrin, isoquercitrin and quercitirin in the crude extract. Gallic acid and ellagic acid were observed in chloroform fraction and in the water fraction ellagic acid, kaempferol, myricitrin and isoquercitrin were detected.
Conclusion: The crude hydromethanolic extract of *P. stellatum* has significant anti-seizure activity. The chloroform and aqueous fractions have antiseizure activity. The extracts have previously identified compounds with anti-convulsant activity which indicates the antiseizure potential of the plant.

1. Introduction

Epilepsy is a debilitating neurological disorder that directly affects approximately 65 million people worldwide (Devinsky et al., 2018). The prevalence of epilepsy in Sub-Saharan Africa is considerably higher than the global average due to high levels of head trauma and brain infections (Preux and Druet-Cabanac, 2005), where active convulsive epilepsy rates can vary between 7 and 14 affected persons per 1000 in rural areas (Ngugi et al., 2013). In addition to this high prevalence, there is also a six-fold increase in mortality in people with epilepsy in Africa (Diop et al., 2005). Even though anti-epileptic drugs are now widely available, many people with active epilepsy go untreated, particularly in

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resource-poor countries. This is largely due to inadequate access to local healthcare workers, the cost of treatment or the unavailability of anti-epileptic drugs. Modern antiepileptic drugs are associated with side effects and in those diagnosed with epilepsy, 30–40% will have seizures, which are resistant to all currently available antiepileptic drugs (Laxer et al., 2014). This underscores the urgent need for the development of novel anti-seizure treatment strategies.

Sub-Saharan Africa includes some 45,000 plant species (Linder, 2014). It is likely that within one of these plants resides the next highly effective antiseizure compound. To expedite the search for novel agents we believe it is logical to make use of Africa's indigenous medical knowledge which remains the prevalent form treatment for convulsive disorders. A survey from a rural African population showed that 42.5% of epileptic children use traditional medicine alone or together with modern medicine (Christianson et al., 2000). Traditional healers in Africa make use of over 150 plant species from at least 63 families for the treatment of epilepsy and convulsions (Ragunathan and Abay, 2009; Seifu, 2014; Stafford et al., 2008; Yineger et al., 2008). Extracts from many species have shown to possess anti-seizure activity (Chauhan et al., 1988; Nsour et al., 2000; Ojewole, 2008; Pedersen et al., 2008; Srinivasan and Roy, 2017). Medicinal plant-derived compounds with antiseizure activities included alkaloids, flavonoids, terpenoids, saponins and coumarins (Zhu et al., 2014).

In Ethiopia, the whole plant juice of *Pterolobium stellatum* (Forssk.) Brenan, a perennial flowering plant in the legume family (Fabaceae), is given orally for one month to treat epilepsy in north-west Ethiopia (Ragunathan and Abay, 2009). This is currently the only available information in the literature pertaining to the preparation, administration, and efficacy of *P. stellatum* and to the best of our knowledge its activity has not been scientifically investigated. This species is widespread along the eastern half of Africa from South Africa in the south and northwards to Ethiopia and the Sudan. Fresh leaves and roots are also chewed for tuberculosis and related respiratory diseases, diarrhoea, and neuralgia (Andualem et al., 2014; Balcha et al., 2014). Very little research has been presented on *P. stellatum's* biological activity or phytochemical constituents. In a recent study on the genotoxicity of Ethiopian medicinal plants, the chloroform extract of *P. stellatum* showed increased tail DNA percentage in a concentration dependent manner (Kahaliw et al., 2018). Considering the strong reliance of many people, especially in most marginalized communities across the globe, on medicinal plants to treat seizures and the global need to discover new, more effective, and safer compounds, the aim of this study was to investigate if there is evidence to substantiate *P. stellatum* use as an anti-seizure agent. This will be achieved by determining whether extracts of *P. stellatum* have antiseizure activity in established *in vitro* and *in vivo* models of seizures and epilepsy. Our objective was to use a crude hydromethanolic (80% methanol) leaf extract of *P. stellatum,* and four fractions of varying polarity, to test for anti-seizure activity in the 0 Mg^{2+}*in vitro* model of seizures as well as in both the maximal electroshock seizure (MES) model and the pentylenetetrazol (PTZ) seizure *in vivo* animal models. A hydromethanolic extract was used instead of the juice of the plant as is traditionally prescribed, since preparation of juice proved difficult to obtain from the material we harvested. Whilst the selected seizure models used in this study are useful in determining anti-seizure activity, they cannot draw any conclusions on the potential mechanisms of action of the extract or fractions.

2. Materials and methods

2.1. Preparation of plant extracts

Leaves from *P. stellatum* were collected in July 2016 from the Awash Melkasa area 50 km south-west of Addis Ababa, Ethiopia. The plants were identified, and a voucher specimen (SS-004) was deposited at the National Herbarium at the Addis Ababa University College of Science. Traditionally, according to one report (Ragunathan and Abay, 2009), the 'plant juice' is used in formulations. However, due to the difficulty in preparation and low 'juice' yield, together with issues of keeping the material fresh after harvesting in the field, it was decided that extraction of dried material, with a polar solvent such as 80% methanol, would be a suitable substitute for preliminary studies. The above ground plant parts (leaves and narrow stems) were collected, mixed, and shade dried in a processing room with an average temperature of 25 °C. This was then powdered and kept at room temperature in a sealed container until extracted. The air-dried and powdered plant material (500 g) of *P. stellatum* were extracted by maceration with 80% methanol for three consecutive days at room temperature. The extract was then filtered and concentrated under vacuum in a rotary evaporator. The residual water was removed by lyophilization using an Operon (Korea vacuum limited, Korea) freeze-dryer. Using this extraction technique 179.51 g was obtained from 500 g of *P. stellatum* dry leaf powder giving a yield of 35.9%. The crude extract of *P. stellatum* was subject to further fractionation to separate compounds by polarity to provide further information on the nature of the active compounds. The dried hydromethanolic powder (50 g) was dissolved in 100 ml warmed distilled water and placed in a separatory funnel, followed by partitioning the extract with 3 × 150 ml pet ether. The pet ether partitions were combined and concentrated providing the pet ether fraction at a yield of 2.2%. The same process was followed with the chloroform and butanol fractions providing yields of 5.8% and 8%, respectively. The final water fraction was obtained by lyophilizing the remaining aqueous residue, resulting in a yield of 29.8% w/w dried material. All the fractions were kept in tightly closed container at −20 °C until used for *in vitro* and *in vivo* test (Debella, 2002). For the 0 Mg^{2+} *in vitro* study the crude extract and fractions were tested at 0.7 mg/ml in triplicate and for the *in vivo* seizure models, the crude extract and fractions were tested at 400 mg/kg and 800 mg/kg.

2.2. Ethics statement

Plant material was collected under the necessary permits held by the National Herbarium at the Addis Ababa University College of Science. All animal handling, care and procedures were carried out in accordance with national (South African National Standard: The care and use of animals for scientific purposes, 2008) and institutional guidelines (OECD. Organisation For Economic Cooperation and Development, 2001). The experimental protocols for *in vivo* experiments were approved by the Institutional Review Board (IRB) of Addis Ababa University (AAU), College of Health Sciences. Approval for the *in vitro* experiments was granted by the University of Cape Town Animal Ethics Committee (Protocol No: AEC 014/035).

2.3. Experimental animals

For the maximal electroshock seizure (MES) model (n = 72) and the pentylenetetrazol (PTZ) seizure model (n = 72) for seizures male BALB/c mice (6–8 weeks old; 20–30 g) were obtained from Ethiopian Public Health Institute. For the toxicity study, female BALB/c mice (n = 6; 6–8 weeks old; 20–30 g) were obtained from Addis Ababa University's College of Health Science Laboratory Animal Unit. For the *in vitro* study C57BL/6 mice (n = 3; P14–P21) were obtained from the University of Cape Town's Research Animal Facility. The extract fractions were not tested in the *in vitro* assay due to the expense and time required to perform these studies. In total 153 mice were used in this study.

2.4. Reagents and chemicals

Pentylenetetrazol (PTZ, Sigma Chemical Co., Sweden), diazepam (Medifarma Pharmaceuticals), sodium chloride ≥99.0% ACS, (VWR Chemicals BDH®, England), petroleum ether, chloroform and n-butanol, methanol (Sigma-Aldrich, Germany), Phenytoin (Brawn Laboratory, India), and Tween 80 (Lobe Chemicals, India), D-glucose (Sigma-Aldrich), NaCl (Sigma-Aldrich). KCl (Sigma-Aldrich), $MgCl_2$ (Sigma-

Aldrich), $CaCl_2$ (Sigma-Aldrich), NaH_2PO_4 (Sigma-Aldrich), $NaHCO_3$ (Sigma-Aldrich), sucrose (Sigma-Aldrich) were obtained from the local suppliers.

2.5. The 0 Mg^{2+} *in vitro* model of seizures

Acute brain slices were prepared from 14- to 21-day-old C57BL/6 mice. After decapitation, the mouse brain was extracted and quickly placed in a 50% sucrose cutting solution bubbled with carbogen gas (95% oxygen and 5% carbon dioxide). The cutting solution used was composed of: NaCl (60 mM); KCl (3 mM); NaH_2PO_4 (1.2 mM); $NaHCO_3$ (23 mM); D-glucose (11 mM); $MgCl_2$ (3 mM); $CaCl_2$ (1 mM) and sucrose (120 mM). The pH was adjusted to between 7.38 and 7.42 using 0.1 mM NaOH. The mouse brain was then appropriately sectioned using a scalpel blade to ensure that the hippocampus and entorhinal cortex would be sliced in the transverse plane. 400 μm horizontal slices were cut using a vibrating VF-200 Compresstome (Precisionary Instruments, USA). This method of preparing acute brain slices is similar to that employed by Dreier and Heinemann (1991) and Dreier et al. (1998a). Slice quality was confirmed by assessing the integrity of the hippocampus and its connection to the entorhinal cortex (EC). The slices were then transferred to a recovery chamber which contained a standard artificial cerebrospinal fluid (aCSF) solution, which was again bubbled with carbogen gas. The standard aCSF solution was composed of: NaCl (120 mM); KCl (3 mM); $MgCl_2$ (2 mM); $CaCl_2$ (2 mM); NaH_2PO_4 (1.2 mM); $NaHCO_3$ (23 mM); D-glucose (11 mM). The slices were kept in the recovery chamber at room temperature (20–25 °C) for a minimum of 40 min before being transferred to the interface rig for local field potential recordings.

The recordings were obtained by placing the slices in an interface recording chamber (Krimer and Goldman-Rakic, 1997) and perfused with aCSF using a peristaltic pump (Model 205S Watson-Marlow, UK). Temperature was adjusted to ensure the solution in the chamber surrounding the slice was kept between 33 and 35 °C. Single-electrode extracellular recordings were performed using glass micropipettes, which were prepared from borosilicate glass capillaries with an outer diameter of 1.20 mm and inner diameter of 0.69 mm (Warner Instruments, USA), using a horizontal puller (Intracell Model P-1000, Sutter, USA).

The tips of the micropipettes were broken under microscope visualisation using a VT-II 2147861 microscope (Olympus, Japan). Pipettes were filled with Mg^{2+}-free aCSF and lowered onto the entorhinal cortex of brain slices under microscope guidance. Once the electrodes were satisfactorily positioned in the tissue, field potential recordings were initiated (Powerlab, AD Instruments). The recordings were verified visually on the LabChart recording software (AD Instruments, Dunedin, New Zealand). Electrical signals were amplified by the Microelectrode AC Amplifier (A-M system, model 1800) with gain set to 10000.

To elicit *in vitro* epileptiform activity, slices were bathed in Mg^{2+}-free aCSF. Removing extracellular Mg^{2+} reduces the voltage dependent block of Mg^{2+} on N-methyl-D-aspartic acid (NMDA) receptors. Initial interictal-like activity is observed followed by the gradual development of seizure-like events (SLEs), which mimic what is observed in temporal lobe seizures in humans (Anderson et al., 1986; Dreier et al., 1998b). Seizure-like events (SLEs) are observable as large, high-frequency events in the local field potential recordings, which lasted more than 5 s. Baseline recordings were made for 600 s with standard aCSF before Mg^{2+}-free aCSF was washed in for 3000 s to induce a seizure-like event. The 0 Mg^{2+} solution (Mg^{2+}-free aCSF) either contained *P. stellatum* crude extract or fractions thereof (0.7 mg/ml), the relevant solvent as a negative control, or diazepam (3 μM) as a positive control. Initially the crude extract was tested (Fig. 1 and Table 1) and once activity was confirmed the four fractions were tested at the same concentration of 0.7 mg/ml (Table 1). The presence of SLEs, time to first SLE, average duration of SLEs and frequency of SLEs was compared between treated slices versus untreated control. The Chi square test with $P < 0.05$ was

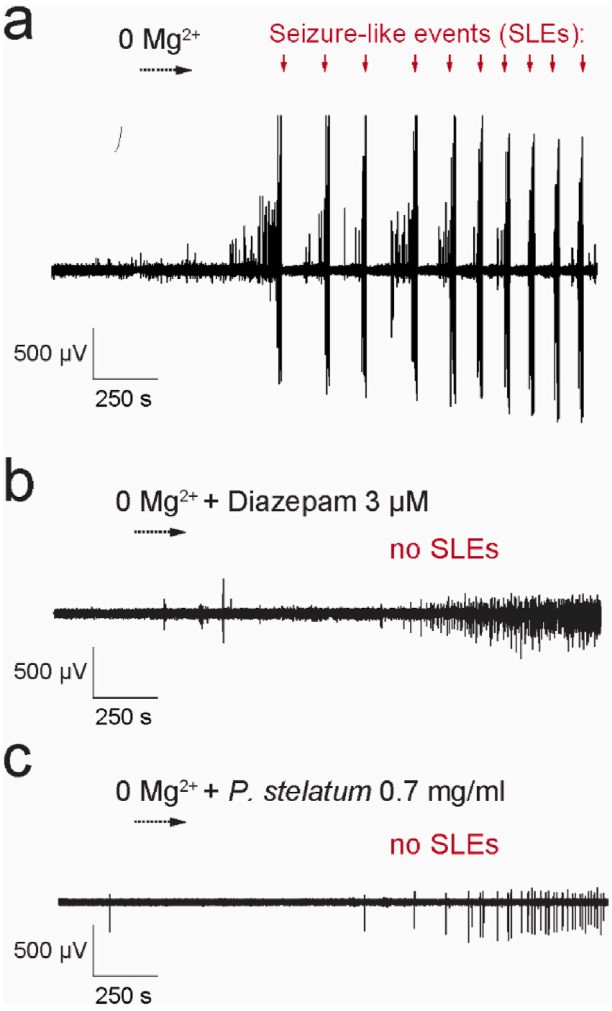

Fig. 1. The *in vitro* 0 Mg^{2+} model of seizures demonstrates the anti-seizure activity of *Pteralobium stellatum*. a) Top trace, a glass electrode is placed in the pyramidal cell layer of a mouse hippocampal brain slice to record extracellular field potentials. Removal of 0 Mg^{2+} from the slice perfusate results in recurrent seizure-like events (red arrows) in control slices. b) Concurrent addition of 3 μM diazepam (a known antiseizure agent) prevented SLE generation. c) Addition of 0.7 mg/ml of crude hydromethanolic *P. stellatum* extract prevented the onset of SLEs in the tested samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Anti-seizure activity of *P. stellatum* extract and fractions (0.7 mg/ml) in the 0 Mg^{2+} *in vitro* seizure model.

Test group	SLE positive	SLE negative	Total No. slices	SLE Protection (%)
Control (solvent)	10	6	16	37.5
Diazepam (3 μM; 0.85 μg/ml)	2	10	12	83.33*
***P. stellatum* crude 80% methanol**	3	13	16	81.25*
***P. stellatum* petroleum ether fraction**	8	8	16	50
***P. stellatum* chloroform fraction**	1	15	16	93.57*
***P. stellatum* butanol fraction**	9	9	18	50
***P. stellatum* water fraction**	0	12	12	100*

* Fishers exact test, significance compared with negative control at $P < 0.05$.
SLE: seizure-like event.

used to determine statistical difference between groups (Qaddoumi et al., 2014; Raimondo et al., 2017).

2.6. Acute toxicity test

Six female BALB/c mice (6–8 weeks old; 20–30 g) were obtained from the AAU College of Health Sciences Laboratory Animal Unit. The mice were kept at room temperature in a humid pathogen-free environment and were exposed to 12 h light and 12 h darkness. The mice were given water and standard food pellets every 24 h. The mice were acclimatized for laboratory condition 48 h before use for the experiments. In order to ascertain a safe oral dose an acute oral toxicity test was performed as per guideline 423 described in the Organisation for Economic Co-operation and Developments guidance document on acute oral toxicity testing (OECD. Organisation For Economic Cooperation and Development, 2001). Six female mice were grouped into two groups randomly using a lottery system (three per group). One group was given distilled water (control) and the other crude extract. The extract was delivered blindly at a dose of 2000 mg/kg.

2.7. In vivo seizure models

Male BALB/c mice weighing between 20 and 30 g were used for both the maximal electroshock seizure (MES) model and the pentylenetetrazol (PTZ) seizure model. Mice were housed under standard conditions at a temperature of 22 ± 2 °C, and with a 12 h light/12 h dark cycle. The mice were provided with free access to a standard pellet laboratory diet and water. The animals were fasted for 4–8 h prior to testing (Hegde et al., 2009) and were acclimatized to the laboratory environment.

2.7.1. The maximal electroshock seizure (MES) in vivo seizure model

The Maximal Electroshock Seizure model is an established model of generalized convulsive seizures used for screening potential anti-epileptic drugs (Kandratavicius et al., 2014). Mice were randomly assigned to 12 groups using a lottery system, ten test groups were administered the crude extract and four fractions (crude hydromethanolic, petroleum ether, chloroform, butanol, and water) at two concentrations and there were two control groups, with six mice in each group. A total of 72 mice were used in this study. Animals in the test groups received 400 mg/kg and 800 mg/kg of each fraction orally. The negative control group received 0.5% Tween 80 (0.3 ml) and a positive control group received phenytoin (10 mg/kg). The animals in all the groups were dosed in a blind manner 1 h before the application of the electric shock. Each animal was securely held and a current of 54 mA was passed for 0.2 s trans-auricularly through ear lobe electrodes using an electroconvulsiometer (Rolex Ambala, India). The duration of hind limb extension was recorded. A reduction in this duration was considered as an anti-seizure action of the agent delivered (Insuasty et al., 2014).

2.7.2. The pentylenetetrazole (PTZ) in vivo seizure model

Pentylenetetrazole is a $GABA_A$ receptor antagonist, once injected it results in generalized convulsive seizures. As a result it is an established method for screening potential anti-epileptic drugs (Kandratavicius et al., 2014). Animals were again randomly assigned to 12 groups and blindly administered treatments as described for the MES test. However, for this test, the positive control group was treated with diazepam (5 mg/kg) orally and not phenytoin. One hour after administering the various agents to the different groups of animals, PTZ (85 mg/kg) was injected subcutaneously, and mice were observed for 30 min for the onset of convulsive behavior. The onset time of convulsions was recorded. A total of 72 mice were used in this study.

2.8. Statistical analysis

GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) and SPSS Statistics 25 (IBM) software were used for analysis. The percentage of protected animals were analysed using the Fisher's Exact Test (two-tail), analyses of variance (ANOVA) with Post Hoc tests (Fisher's LSD) were performed with SPSS version 25.

2.9. Characterisation of plant extract and fractions

The plant extract and respective fractions were characterised utilising a Waters SYNAPT G2 ultraperformance liquid chromatography-mass spectrometer (UPLC-MS) (Waters Corporation, Massachusetts, USA) system. The samples were dissolved in methanol to obtain a final concentration of 1 mg/ml of which 5 µl per sample was injected into the system using a Acquity autosampler (Waters Corporation, Massachusetts, USA). The compounds were separated on the LC system using a binary solvent (A: H_2O + 0.1% H_2CO_2, B: MeOH + 0.1% H_2CO_2) gradient at a flow rate of 0.3 ml/min coupled to an Acquity UPLC HSS T3 Column (100 Å, 1.8 µm, 2.1 mm × 150 mm, Waters Corporation, Massachusetts, USA) set at 50 °C. Solvent B was set to an initial concentration of 3% with a gradual increase to 100% over 14 min and held for 2 min followed by a rapid decrease to 3% over 50 s, held until the final run time of 20 min. The MS related total ion current (TIC) chromatograms and associated mass hertz (m/z) fragmentation patterns were generated using positive (ES^+)- and negative (ES^-) ionisation modes at a sample infusion rate of 10 µl/min. The mass range of 50–1200 Da was assessed using helium as desolvation gas at a flow rate of 400 l/h. The ion source and desolvation temperatures were set to 120 °C and 300 °C, respectively. The MS collision energies (eV) and capillary voltages (kV) were set to 4eV, 2.6 kV (ES^+) and 6eV, 2 kV (ES^-), respectively. Data interpretation was done using MestReNova version 14.2 in combination with the National Institute of Standards and Technology (NIST) version 2.2 MS database (NIST 14, Agilent Technologies, USA).

3. Results

3.1. Extracts of P. stellatum have anti-seizure activity in the 0 Mg^{2+} in vitro model of seizures

Removal of 0 Mg^{2+} from the slice perfusate resulted in the onset of recurrent seizure-like events (SLEs) in 10 of the 16 control slices tested (see Fig. 1a and Table 1), which occurred 1774 ± 228 s after the wash in of 0 Mg^{2+}aCSF. We first confirmed the sensitivity of our preparation to the known anti-seizure benzodiazepine agent diazepam. The addition of 3 µM diazepam to the 0 Mg^{2+}aCSF significantly reduced the number of slices which generated SLEs ($P = 0.024$, Fishers exact test, see Fig. 1b and Table 1). Thereafter, 0.7 mg/ml crude hydromethanolic extract was added to the 0 Mg^{2+}aCSF. This, with similar efficacy to 3 µM diazepam, significantly prevented SLE generation ($P = 0.029$, Fishers exact test, see Fig. 1c and Table 1).

Given the anti-seizure efficacy of *P. stellatum* crude hydromethanolic extract in the 0 Mg^{2+} model of seizures when testing the various fractions of the crude extract, we found that both the chloroform and water extracts were highly effective and preventing the induction of seizure-like activity (Table 1). In contrast, the petroleum ether and the butanol extracts had no discernible anti-seizure activity (Table 1). These *in vitro* results suggest that multiple compounds in *P. stellatum* likely have anti-seizure activity.

3.2. P. stellatum extract is not acutely toxic when delivered at 2000 mg/kg in vivo

For the acute toxicity test animals were given *P. stellatum* crude hydromethanolic extract at a dose of 2000 mg/kg. The animals were carefully monitored for 14 days followed by humanely killing the animals using barbiturates and performing post-mortem examinations. We observed no discernible behavioral changes in the animals following oral dosing with *P. stellatum* nor could we observe any changes on post-

mortem examinations. The results of the preliminary acute toxicity test suggest that at up to doses of 2000 mg/kg *P. stellatum* crude extract is not acutely toxic.

3.3. *P. stellatum extracts have anti-seizure activity in the in vivo maximal electroshock seizure model*

After observing *in vitro* anti-seizure effects of the crude extract of *P. stellatum* as well as its chloroform and water fractions, we next sought to determine whether these also had anti-seizure activity *in vivo*. Two of the six control mice survived electrical stimulation in contrast to a dosage with 10 mg/kg of phenytoin, an established anti-seizure agent, resulting in all the mice surviving treatment and an abolishment of hindlimb extension. The crude hydromethanolic extract of *P. stellatum* at two doses (400 mg/kg and 800 mg/kg) improved animal survival in a dose dependant manner. In addition, the crude extract also significantly reduced hind limb extension time (Table 2). The plant extract and fractions protected against seizure development in some animals (16.7–33.3%). This demonstrated an anti-seizure property of the crude extract of *P. stellatum* in the MES model, however it should be noted that phenytoin showed much greater protection (100%) and that none of the extracts showed complete protection from electroshock induced seizure.

Given the efficacy of the crude *P. stellatum* extract in the MES model we next tested the various fractions of the crude extract and fractions (see Materials and Methods). We found that both doses (400 mg/kg and 800 mg/kg) of the *P. stellatum* chloroform fraction significantly reduced the duration of hind limb extension as did the 800 mg/kg water fraction of *P. stellatum* (Table 2). The chloroform and water fractions also appeared to improve animal survival. The petroleum ether and butanol fractions had no significant effect on seizure duration as measured by hind limb extension time (Table 2). These findings demonstrate that the crude extract of *P. stellatum* has *in vivo* anti-seizure activity as demonstrated by the MES model. In addition, the same fractions with *in vitro* anti-seizure activity also had anti-seizure activity in the MES model.

3.4. *The extract of P. stellatum shows anti-seizure activity in the in vivo pentylenetetrazole model of seizures and epilepsy*

The time to the first myoclonic seizure was measured. Control animals experienced myoclonic seizures in comparison the anti-seizure drug diazepam (5 mg/kg), which did not experience seizures, during the 30 min observation period. Dosing the animals with either 400 mg/kg or 800 mg/kg of crude *P. stellatum* extract significantly increased the time to myoclonic seizure onset in a dose-dependent manner (Table 3).

Finally, we tested the different fractions of *P. stellatum* extract in the *in vivo* PTZ model. At both 400 mg/kg and 800 mg/kg dosages, the chloroform and water fractions significantly delayed the onset of myoclonic seizures (Table 3). In contrast, at all dosages, the petroleum ether and butanol fractions had no significant effect on the latency to myoclonic seizures compared with the negative control (Table 3). The results from the PTZ *in vivo* seizure model therefore corroborate those of the MES model and the *in vitro* 0 Mg^{2+} model in confirming the anti-seizure activity of the crude extract of *P. stellatum* as well as the chloroform and water fractions. In this study the tonic-clonic seizures were significantly delayed in all the test extracts except the doses of the petroleum ether fraction compared with the negative control. Again, it should be noted that the positive control, diazepam provided complete protection from seizures whereas of the plant extracts only the crude extract and water fraction provided only partial protection (16.7 and 33.3%, respectively).

3.5. *Characterization of the plant extract and respective fractions*

The stacked UPLC-MS total ion current (TIC) chromatograms (Fig. 2) indicate the differences in chemical constituents of the different fractions compared to the total crude hydromethanolic extract and solvent

Table 2
The crude 80% methanol extract and fractions of *P. stellatum* extract anti-seizure activity in the MES model.

Treatment groups	n	Mean Hindlimb Extension time (s)[a]	Survival rate	Protection from seizure (%)
Negative Control	6	24.33 ± 2.45	2/6	0
80% methanol 400 mg/kg	6	13.00 ± 2.61[b]	5/6	16.67
80% methanol 800 mg/kg	6	11.17 ± 4.09[b]	4/6	33.33
Petroleum ether 400 mg/kg	6	24.00 ± 2.68	3/6	0
Petroleum ether 800 mg/kg	6	18.50 ± 1.78	2/6	0
Chloroform 400 mg/kg	6	15.50 ± 1.88[b]	5/6	0
Chloroform 800 mg/kg	6	11.50 ± 4.29[b]	5/6	33.33
Butanol 400 mg/kg	6	22.50 ± 1.23	4/6	0
Butanol 800 mg/kg	6	17.67 ± 3.67	5/6	0
Water 400 mg/kg	6	20.17 ± 1.85	4/6	0
Water 800 mg/kg	6	13.67 ± 2.96[b]	5/6	16.67
Phenytoin 10 mg/kg	6	0 ± 0.00	6/6	100

[a] Mean ± standard error of mean (SEM).
[b] ANOVA test compared with negative control, significance at $P < 0.05$, $F(11,60) = 7.64$.

Table 3
The crude 80% methanol extract and fractions of *P. stellatum* extract anti-seizure activity in the PTZ seizure model.

Test group	n	Mean latency to myoclonic seizures (s)[a]	Mean latency for tonic-clonic seizure (s)[a]	Protection from seizures (%)
Negative Control	6	239.67 ± 33.72	449.50 ± 70.13	0
80% methanol extract 400 mg/kg	6	542.50 ± 94.03*	1331.50 ± 177.34*	0
80% methanol *extract* 800 mg/kg	6	809.17 ± 225.67*	1198.67 ± 227.25*	16.67
Petroleum ether fraction 400 mg/kg	6	429.50 ± 98.90	479.17 ± 111.45	0
Petroleum ether fraction 800 mg/kg	6	321.17 ± 33.93	472.67 ± 83.71	0
Chloroform fraction 400 mg/kg	6	657.00 ± 91.62*	956.17 ± 201.18*	0
Chloroform fraction 800 mg/kg	6	659.83 ± 160.39*	1158.00 ± 223.49*	0
Butanol fraction 400 mg/kg	6	423.50 ± 61.70	994.83 ± 187.28*	0
Butanol fraction 800 mg/kg	6	446.17 ± 85.93	1075.67 ± 241.00*	0
Water fraction 400 mg/kg	6	972.33 ± 276.04*	1259.67 ± 179.21*	33.33
Water fraction 800 mg/kg	6	653.50 ± 116.78*	941.33 ± 193.15*	0
Diazepam 5 mg/kg	6	1800.00 ± 0.00*	1800.00 ± 0.00*	100

*ANOVA test compared with the negative control with significance at $P < 0.05$, $F(11,60) = 5.279$.
[a] Mean ± standard error of mean (SEM).

Table 4
UPLC-MS generated chemical properties of the highlighted peaks represented in Fig. 2

Previously identified compound from *P. stellatum* (chemical formula; monoisotopic mass)	Retention time (min) of identified compound/derivative in crude hydromethanolic extract[a]	Observed mass (m/z+ [M-H]$^-$) ± error (Da)	Major mass fragments- and compound *match* ion (ES$^-$ (m/z))	MS purity &TIC Peak purity (crude hydromethanolic extract)	MS purity & TIC Peak purity (fractions)[b]
1.Gallic acid ($C_7H_6O_5$; 170.02)	Rt 3.88	169.01 ± 4.0 × 10^{-5}	125.04; ***169.01***; 343.07; 546.99	MS 48.40% TIC 35.63%	MS P - 45.2% C - 31.3% B - 53.2% W - ND TIC P - 28.91% C - 45.0% B - 28.57% W - ND
2. Ellagic acid ($C_{14}H_6O_8$; 302.01)	Rt 5.01	301.06 ± 5.8 × 10^{-3}	149.05; 169.05; ***301.06***; 603.12	MS 30.40% TIC 13.45%	MS P - 29.8% C - 9.8% B - 29.6% W - 30.2% TIC P - 15.56% C - 55.0% B - 11.85% W - 62.33%
3. Kaempferol ($C_{15}H_{10}O_6$; 286.05)	Rt 5.59	285.06 ± 2.1 × 10^{-3}	169.01; ***285.06***; 353.04; 495.08	MS 33.90% TIC 4.23%	MS P - 32.2% C - ND B - ND W - 38.9% TIC P - 5.11% C - ND B - ND W - 27.30%
4. Myricitrin ($C_{21}H_{20}O_{12}$; 464.09)	Rt 8.82	463.09 ± 2.7 × 10^{-5}	183.03; ***463.09;*** 609.15; 761.16; 927.18	MS 33.60% TIC 14.61%	MS P - 36.3% C - ND B - 36.3% W - 12.5% TIC P - 15.95% C - ND B - 15.40% W - 5.18%
5. Isoquercitrin ($C_{21}H_{20}O_{12}$; 464.09)	Rt 8.82	463.09 ± 2.7 × 10^{-5}	183.03; ***463.09;*** 609.15; 761.16; 927.18	MS 33.60% TIC 14.61%	MS P - 36.3% C - ND B - 36.3% W - 12.5% TIC P - 15.95% C - ND B - 15.4% W - 5.18%
6. Quercitirin ($C_{21}H_{20}O_{11}$; 448.10)	Rt 9.11	447.09 ± 1.0 × 10^{-4}	183.03; ***447.09***; 593.15; 895.19; 1041.25	MS 29.40% TIC 17.40%	MS P - 29.4% C - ND B - 29.4% W - ND TIC P - 18.51% C - ND B - 16.13% W - ND

The major fragments were identified using the MestReNova MS prediction algorithm in conjunction with the NIST 14 MS/MS spectral library database.
ND- Not Detected and/or Match score below 95% and/or Retention time (Rt) incorrect.
[a] The retention times presented in the table refer only to the negative ionisation mode data of the crude hydromethanolic extract.
[b] The fractions are described as; P- petroleum ether, C- chloroform, B- n-butanol, W- water

blank. The highlighted peaks are of interest as the corresponding MS fragmentation patterns correspond to that of previously identified compounds from *P. stellatum*. These compounds could be the major contributors to the *in vitro* and *in vivo* results presented, as their previously reported biological activity indicate antiseizure and anti-epileptic activity.

The chemical properties of the identified peaks in Fig. 2 are presented in Table 4. The accuracy of prediction was based on sample-to-database MS fragmentation pattern comparisons as well as correlating the relative retention time of the compounds to their solubility in the solvents used during the LC separation. Based on fragmentation pattern analyses, all the previously identified compounds were present in the

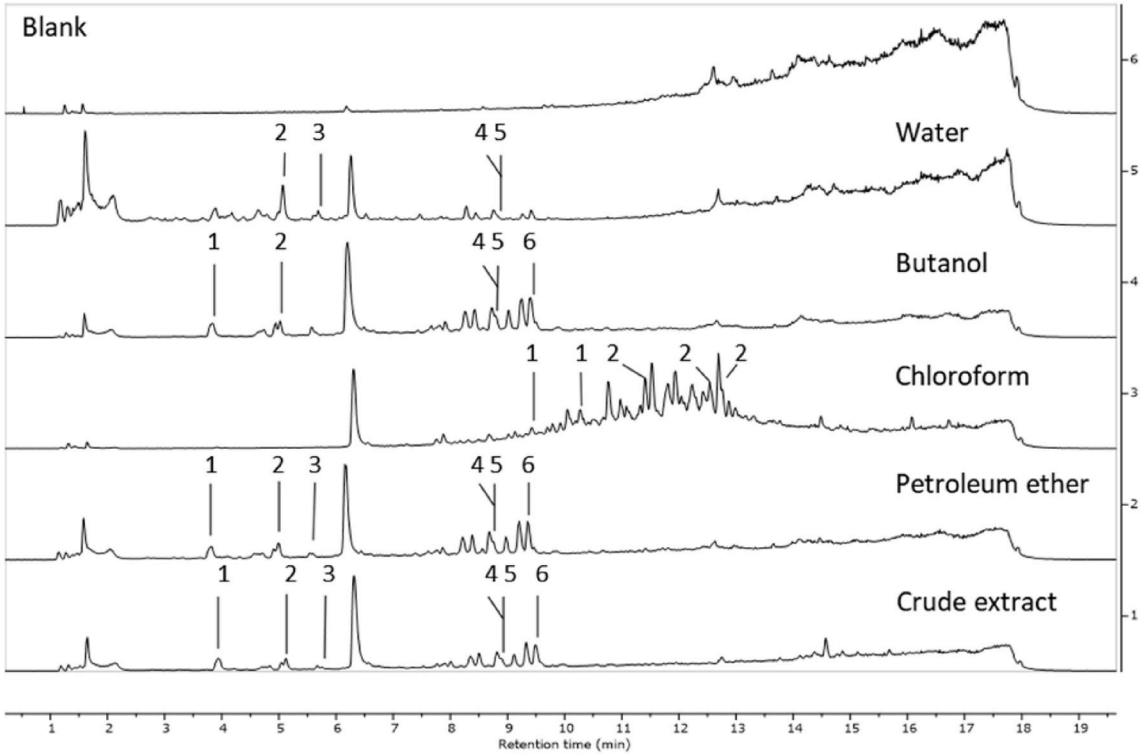

Fig. 2. The negative mode ionisation (ES^-) stacked UPLC-MS TICs of *P. stellatum* crude hydromethanolic extract and respective fractions. The stacked chromatograms were generated using MestReNova analytical software. The highlighted peaks represent previously identified compounds- or derivatives from *P. stellatum* which are unique to the plant extract and not found in the solvent blank. These compound peaks are described in Table 4.

extracts, however, fluctuations in qualitative concentrations were observed in the different fractions. The low MS purity values indicate that either optimal compound separation was not achieved or that these compounds contribute as precursors of larger chemical structures, as is the case with the derivative of gallic acid, gallic acid 4-O-(6-galloylglucoside) both of which were identified in the chloroform fractions (derivative data not shown), or a combination of both these factors. The relatively low collision energies used during data acquisition allowed most compounds to be preserved with very little product ion formation (Kind et al., 2017). This allowed for fast screening of the samples based on the molecular mass of the compounds adjusted according to the addition or loss of specific anions or cations, respectively. However, because of the limited fragments that can be compared between the observed fragmentation pattern and the predicted library patterns, care should be taken as misidentification can occur. Thus, by combining the fragmentation patterns with other chemical properties such as the solubility and polar surface area we can predict the order in which the compounds elute from the LC column, thereby providing greater confidence in the assigned compounds.

4. Discussion

In this study we investigated the anti-seizure properties of leaf extracts of the Ethiopian plant *P. stellatum*, and the various fractions thereof. We found significant and consistent anti-seizure activity of the crude extract in the *in vitro* 0 Mg^{2+} model of seizures as well as in two established *in vivo* models of seizures. It showed significant effect in reducing the hindlimb extension time the MES model and with the PTZ model it improved the mean latency of myoclonic seizure as well as tonic-clonic seizures. Furthermore, across all three models we found anti-seizure activity in the chloroform and aqueous fractions of the extract. To our knowledge this is the first demonstration of the anti-seizure properties of extracts of *P. stellatum*.

P. stellatum has been used as a traditional medicine to treat epilepsy and convulsions in Ethiopia (Ragunathan and Abay, 2009), which provided the rational for our efforts to determine its anti-seizure activity using established models of epilepsy. The 0 Mg^{2+} model is a well-established *in vitro* model of seizures, which is excellent at identifying agents which are capable of reducing the onset and propagation of seizures (Dulla et al., 2018). The MES model is the most widely used *in vivo* animal model utilised in antiepileptic drug discovery because the predictive value for detecting clinically effective antiepileptic drugs is high. It is thought to be well suited for identifying compounds with activity against generalized tonic-clonic seizures and partial seizures (Pitkanen et al., 2006). It has had particular success in identifying compounds which inhibit Na^+ channels as their primary mechanism of action, such as phenytoin (Pitkanen et al., 2006). Similarly, the PTZ model has been useful, and is thought to be suitable for identifying agents with efficacy against generalized myoclonic and absence seizures (Pai et al., 2012). All of the currently available anti-epileptic agents used clinically have activity in either one or both of the MES or PTZ models (Rho et al., 2010). The fact that extract of *P. stellatum* has significant anti-seizure activity across both models, and at doses which were not acutely toxic, supports the potential utility of compounds contained there-in for possible clinical use in the treatment of epilepsy.

Whilst we have elucidated clear anti-seizure activity of *P. stellatum* using three different models of seizures, we cannot draw any conclusions on the potential mechanisms of action of the active compound or compounds. Anti-seizure activity could be a result of multiple different mechanisms including positive modulation of GABA receptors (as is the case for benzodiazepines), antagonism of glutamate receptors (felbamate and perampanel) or reduction of voltage gated cationic conductance (phenytoin and valproate) (Pitkanen et al., 2006). Future work could seek to determine the mechanism of action of the active components of *P. stellatum* using radiolabelled ligand and binding studies (Risa et al., 2004; Stafford et al., 2005), patch-clamp electrophysiology or calcium imaging (Marchetti et al., 2011).

The UPLC-MS analysis in the current study also indicated the

presence of gallic acid, ellagic acid, kaempferol, myricitrin, isoquercitrin and quercitirin in the crude extract. Of these gallic acid and ellagic acid were found in chloroform fraction. In the water fraction ellagic acid, kaempferol, myricitrin and isoquercitrin were found. These show some of the metabolites tend to concentrate in certain fractions which may contribute to the activity of the chloroform and water fractions.

High-performance liquid chromatography and thin-layer chromatography has previously been used on aqueous acetone extracts of leaves of *P. stellatum* to demonstrate that the leaves contain high levels of phenolic compounds. These include gallic acid, ellagic acid and flavonoids or flavonoid derivatives, namely isoquercitrin, quercitrin, myricitrin and kaempferol (Mueller-Harvey et al., 1987). Many of the compounds have previously been demonstrated to possess anti-seizure activity in animal models. For example ellagic acid is known to have anti-seizure activity in the PTZ model of seizures (El-Missiry et al., 2020). In addition, there is considerable evidence linking many flavonoids with anti-seizure activity. This is thought to be due to the structural similarity between flavonoids and benzodiazepines and hence positive modulation of the $GABA_A$ receptors (Choudhary et al., 2011; Kavvadias et al., 2004). Isoquercitrin, myricitrin and kaempferol are such examples and have all shown anti-seizure activity (Copmans et al., 2018; Orhan et al., 2012; Sun et al., 2019). If the active anti-seizure compounds are shown to act against $GABA_A$ receptors it is worth noting that they may suffer from similar issues to benzodiazepines in terms of their utility for treating epilepsy. This includes the potential for side-effects, especially sedative effects, and the high risk of the development of tolerance. Having the isolated compounds with literature support of anti-seizure activity the clear evidence for the anti-seizure effects of *P. stellatum* shown here warrants further investigation, particularly into the efficacy and mechanism of action of the compounds identified.

5. Conclusions

Our findings suggest that *P. stellatum* is likely to contain compounds with anti-seizure activity. The active compounds may be concentrated in both the chloroform and water-based fractions of the crude leaf extract, based on their anti-seizure activity. Future work should seek to isolate single compounds, notably those already identified in this study, for efficacy and toxicity studies. Finally, extracts of *P. stellatum* should be tested in more chronic models of epilepsy to determine whether *P. stellatum*-derived compounds could have anti-epileptogenic as well as anti-seizure properties.

In conclusion this study provides preliminary scientific rationale for the use of the plant extract for the amelioration of seizures as observed as part of traditional medicine practices in Ethiopia. Furthermore, it identifies *P. stellatum* as a promising plant containing molecules with anti-seizure potential for further isolation of single compound and test for efficacy, toxicity as well as study their mechanism of action.

CRediT authorship contribution statement

Samson Sahile Salile: Formal analysis, Writing – original draft, Formal analysis, Investigation. **Hamin John Lee:** Formal analysis, Methodology, Visualization, Formal analysis. **Paul Sewes Frederick Alberts:** Methodology, Writing – review & editing, Visualization, Formal analysis. **Teferra Abula:** Conceptualization, Funding acquisition, Supervision. **Joseph Valentino Raimondo:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision. **Gary Ivan Stafford:** Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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