



BJP

Bangladesh Journal of Pharmacology

Research Article

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Article Info

Received: 11 March 2019
Accepted: 12 December 2019
Available Online: 15 December 2019
DOI: 10.3329/bjp.v14i4.40537

Cite this article:

Bezsa K, Gabbas ZE, Laadraoui J, Laaradia MA, Oufquir S, Chait A. Ameliorative potential of *Anacyclus pyrethrum* extract in generalized seizures in rat: Possible cholinergic mediated mechanism. Bangladesh J Pharmacol. 2019; 14: 188-95.

Abstract

The present study evaluates the anticonvulsant activity of the roots of *Anacyclus pyrethrum* using pilocarpine-induced experimental model of epilepsy in rat, and to determine its possible anticonvulsant mechanism. Ethanol extract (200 and 400 mg/kg) or alkylamides (25 and 50 mg/kg) was administered orally 45 min before the injection of pilocarpine-induced (400 mg/kg) seizures. The possible anticonvulsant mechanism was investigated by testing the effect of atropine (2 mL/kg) and scopolamine (1 mg/kg). The scoring of seizure severity, seizures time latency, duration of total seizures and percentage of mortality protection were recorded. Ethanol extract and alkylamides prolonged the time of onset seizure and decreased the duration of seizures compared to control group ($p < 0.001$). The seizure protection was 100%. The co-administered of ethanol extract of *A. pyrethrum* and alkylamides with atropine completely abolished the pilocarpine-induced seizures.

Introduction

Epilepsy is known as one of the most prevalent and severe neurological disorders. There are almost one million deaths annually as a consequence of epilepsy (Bell et al., 2014; Hauser et al., 1993).

Antiepileptic drugs are prescribed alone or in combination as a means of curing epilepsy (Hernan and Holmes, 2016). These antiepileptic drugs, in most cases, cause one or more secondary effects (Kaur et al., 2016).

To overcome these problems, several studies conducted in recent years have supported the use of herbal remedies as alternatives. To this end, animal studies have shown that several plants such as *Abies webbiana* (Parkash et al., 2015), *Artemisia persica* (Daneshkhah and Setorki, 2019), *Carum carvi* (Showraki et al., 2016), *Citrus sinensis* (Citraro et al., 2016), *Crocus sativus* (Khazdair et al., 2015), *Dodonaea viscosa* (Karim et al., 2015),

Kalanchoe pinnata (Mora-Pérez and Hernández-Medel, 2016), *Laggera aurita* (Malami et al., 2016), and *Silybum marianum* (Waqar et al., 2016) have anticonvulsant effect over experimental models of epilepsy.

Anacyclus pyrethrum, L. (Asteracea) is used to treat a diverse range of diseases. Alkamide derivatives have antiprotozoal activity *in vitro* (Althaus et al., 2014). It has tonic effect on the nervous system (Nadkarni, 2005) and possesses potent anticonvulsant activity against experimental model of epilepsy (Pahuja et al., 2012; Zaidi et al., 2009). According to Veryser et al. (2016), pellitorine readily absorbed from the intestine and is rapidly penetrated into the blood-brain barrier. Therefore, this compound may have a potential therapeutic role in the treatment of central nervous system diseases. Extracted roots possess antimicrobial, saliva stimulation, local anaesthetic, insecticidal, and anti-depressive effects. They are mainly designed to treat rheumatism,



paralysis, toothache, and epilepsy (Annalakshmi et al., 2012; Boonen et al., 2012). Aqueous extracts are non-toxic and are being used as aphrodisiacs to improve male libido (Sharma et al., 2009; Sujith et al., 2012). The animal study showed that *A. pyrethrum* root extract might produce an antidepressant effect (Badhe et al., 2010). The present study investigates the anticonvulsant activity and possible mechanisms of action of *A. pyrethrum* in pilocarpine-induced seizures in rats.

Materials and Methods

Animal

The male Sprague Dawley Sprague rats weighing 180 to 230 g were placed in a room at a temperature of $22 \pm 2^\circ$ C under a cycle of 12:12 hours light:dark with free access to water and food. The animals were provided by the Animal Care Facility of the Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco. Animals were housed in groups of six rats per standard Makrolon cage. The rats were allowed to acclimatize to the housing room conditions for 24 hours before behavioral procedures were initiated. All experiments were performed between 11:00 AM and 5:00 PM. Each animal was used only once to reduce the number of animals used in the experiments.

Plant material and preparation of extracts

The root of *A. pyrethrum* was collected in May 2014 from Bin El Ouidan, Morocco. The identification was performed by the Prof. Ouhammou of the Department of Biology, Faculty of Sciences-Semlalia, Cadi Ayyad University. A voucher specimen MARK-10003 was deposited in the Herbarium of the Department of Biology.

The dried roots were crushed to obtain a powder. Then, ethanol solvent (600 mL) was added to 200 g powder to obtain the extract using the soxhlet apparatus. Ethanol was completely evaporated using a rotary evaporator. The weight of the concentrated extract was 34.1 g, representing about 17.1% yield.

Extraction of alkylamides of *A. pyrethrum*

The powder (500 g) was extracted with 95% ethanol in a soxhlet extractor for 20 hours to complete exhaustion. The extract was collected and dried under vacuum using a rotavapor (Stuart RE300, Bibby Scientific, UK).

The ethanol extract was dissolved in distilled water. The aqueous phase was extracted with chloroform. The chloroform extract was collected and dried under vacuum using a rotavapor. The weight of the chloroform extract was 9.2 g representing about 1.8% yield.

Acute and subchronic toxicity

Mice (25–35 g) were equally divided into 10 groups (six animals per group). They were orally treated by

gavage with different doses (200, 400, 1000, 2000, 5000 mg/kg) of ethanol extract and (25, 50, 100, 1000, 2000 and 5000 mg/kg) of alkylamides solution for the acute toxicity. Mice were observed during the first 12 hours after administration of the drug to detect signs of toxicity and health.

In the subchronic toxicity, four groups were treated daily with ethanol extract (200 and 400 mg/kg) and alkylamides (25 and 50 mg/kg) at 10 mL/kg. A control group received distilled water only. Mice were observed daily and weighted for 14 and 90 days following treatment.

After that period, mice were sacrificed by cervical dislocation, blood was collected and vital organs were immediately collected then weighed, in order to perform biochemical analysis and histological studies.

Histological study

The vital organs such as liver, heart, lungs, and kidneys were dissected and fixed in 10% formalin solution for one night. After the organs are subjected to dehydration in a series of grade alcohols and incorporated into paraffin wax. Sections of 4–10 μ m thickness were stained with hematoxylin and eosin for histological studies (Kiernan, 1999).

Biochemical analysis

Biochemical analysis was carried out on the serum of mice. Serum was obtained by centrifugation of blood samples. Creatinine, aminotransferases, creatine phosphokinase and lactate dehydrogenase levels were quantified in the control and treated groups, using the standard method with a biochemical automat (C311 ROCH).

Characterization of *A. pyrethrum* extract

The *A. pyrethrum* root extract was prepared as previously described (Boonen et al., 2012). For the analytical characterization of the extract, it was dissolved in ethanol, vortexed, sonicated for 2 hours and centrifuged for 15 min at $3,220 \times g$ at room temperature. A 30:70 water/ethanol (v/v) solution was prepared, centrifuged again and the supernatant was filtered using a 0.45 μ m nylon HPLC filter (Whatman).

The high-performance liquid chromatography (Knauer) equipped with a pump (K-1001) and a PDA detector (200–700 UV-Vis) was used to separate and characterize compounds present in *A. pyrethrum* extract. The analysis was performed with a Eurospher II 100-5, column (4, 6 \times 250 mm), and the temperature was set at 25°C. The flow rate was 1 mL/min, the sample volume injected was 2 μ L, and the wavelength of detection was set at 258 nm. 1% acetic acid in water (A) and acetonitrile (B) mixture were used as the optimal mobile phase for a total running time of 166 min. A gradient with a flow rate of 1.0 mL/min was used as follows: t =

0 min: 80:20 A:B (v/v); t = 0–150 min: 10:90 A:B (v/v); t = 150–151 min: 80:10 A:B (v/v); t = 151–166 min: 80:20 A:B (v/v); t = 166 min: 80:20 A:B (v/v).

Drug treatment

Atropine, scopolamine, diazepam and pilocarpine were purchased from the Sigma-Aldrich (France). Atropine (2 mL/kg), scopolamine (1 mg/kg), diazepam (4 mg/kg) and pilocarpine (400 mg/kg) were injected intraperitoneally. The ethanol extract (200, 400 mg/kg) and alkylamides (25, 50 mg/kg) were administered orally.

Treatment

Animals were divided into 16 groups of six animals each. Pilocarpine (400 mg/kg), pilocarpine plus ethanol extract of *A. pyrethrum*, pilocarpine plus alkylamides, pilocarpine plus ethanol extract and atropine, pilocarpine plus ethanol extract and scopolamine, pilocarpine plus alkylamides and atropine, pilocarpine plus alkylamides and scopolamine, pilocarpine plus atropine, pilocarpine plus scopolamine and pilocarpine plus diazepam. The ethanol extract and alkylamides were administered orally by gavage 45 min, while atropine, scopolamine (cholinergic antagonist) and diazepam were injected 30 min before injection of pilocarpine. All drugs were orally administered between 9:00 AM and 12:00 AM.

Epileptic behavior testing

After the intraperitoneal injection of pilocarpine to all groups, each rat was placed in the center of the glass cage, and its behavior was observed and monitored within a 90 min period. The observed epileptic behaviors were ranked as follows: Latency of seizure (min), the latency of tonic-clonic seizure (sec), duration of tonic-clonic seizure (sec), duration of seizures (min), and mortality protection (%).

The scoring of seizures severity after the injection was recorded during the observation period (90 min) according to the scale: 0: Normal activity; 1: Immobility and/or staring; 2: Rigidity, tail extension, head bobbing; 3: Repetitive movements, bilateral pawing, rearing, hind limb tremors; 4: Minor seizure or wobbling, jumping, falling; 5: Tonic-clonic convulsions or multiple and/or occurrence of rating 4; 6: Severe tonic-clonic seizure; 7: Death.

Percentage of seizure protection

The percentage of protection is calculated using the equation given below. This protection is manifested when the animal returns to normal and gains its normal exploratory behavior.

$$100 - \left[\left(\frac{\text{No. of animals showed seizures}}{\text{Total no. of animals used}} \right) \times 100 \right]$$

Statistical analysis

Comparisons between treatment groups and control

were performed by one-way analyses of variance (ANOVA) followed by Tukey's HSD test or Dunnett's test, when appropriate. A value of $p < 0.05$ was considered to be significant.

Results

Histopathological and biochemical analysis of toxicity

No visible sign or symptom of toxicity in mice was observed after oral administration of *A. pyrethrum* ethanol extract. The maximum dose used was 5000 g/kg. Also, no mortality or significant changes in body weight or organ weight was observed 14 days after administration of ethanol extract.

In the subchronic study, mice received ethanol extract and alkylamides for 90 days with no resulting mortality or signs of toxicity. There were no changes in body and organ weights. Histological examination has not revealed any macroscopic or microscopic tissue lesions.

Creatinine, alanine transaminase, lactate dehydrogenase, aspartate aminotransferase, and creatine phosphokinase levels were also not affected (data not shown).

Scoring of seizure severity

There was a significant decrease in the seizure score in the different groups of rats pretreated with alkylamides, ethanol extract, (alkylamides plus scopolamine), (ethanol extract of *A. pyrethrum* plus scopolamine), and diazepam compared to that in the pilocarpine-treated group. Noteworthy, the (alkylamides plus atropine), (ethanol extract of *A. pyrethrum* plus atropine) has completely abolished the pilocarpine-induced convulsions (Figure 1 (A, B)).

Seizures time latency

The treatment with ethanol extract of *A. pyrethrum* (200; 400 mg/kg p.o), alkylamides (25; 50 mg/kg), (alkylamides plus scopolamine), (ethanol extract plus scopolamine), (alkylamides plus atropine), (ethanol extract plus atropine) and, diazepam increased significantly the latency time as compared to the pilocarpine-treated groups ($p < 0.001$) (Figure 1 (C, D)).

Duration of total seizures

The duration of total seizures was significantly higher in pilocarpine-treated group compared to all treated groups with alkylamides, ethanol extract, (alkylamides plus scopolamine), (ethanol extract plus scopolamine), (alkylamides plus atropine), (ethanol extract plus atropine) and diazepam (Figure 1 E, F).

Rate of tonic-clonic seizures

No tonic-clonic seizure was observed in all the treated groups compared to the pilocarpine-induced convulsion models.

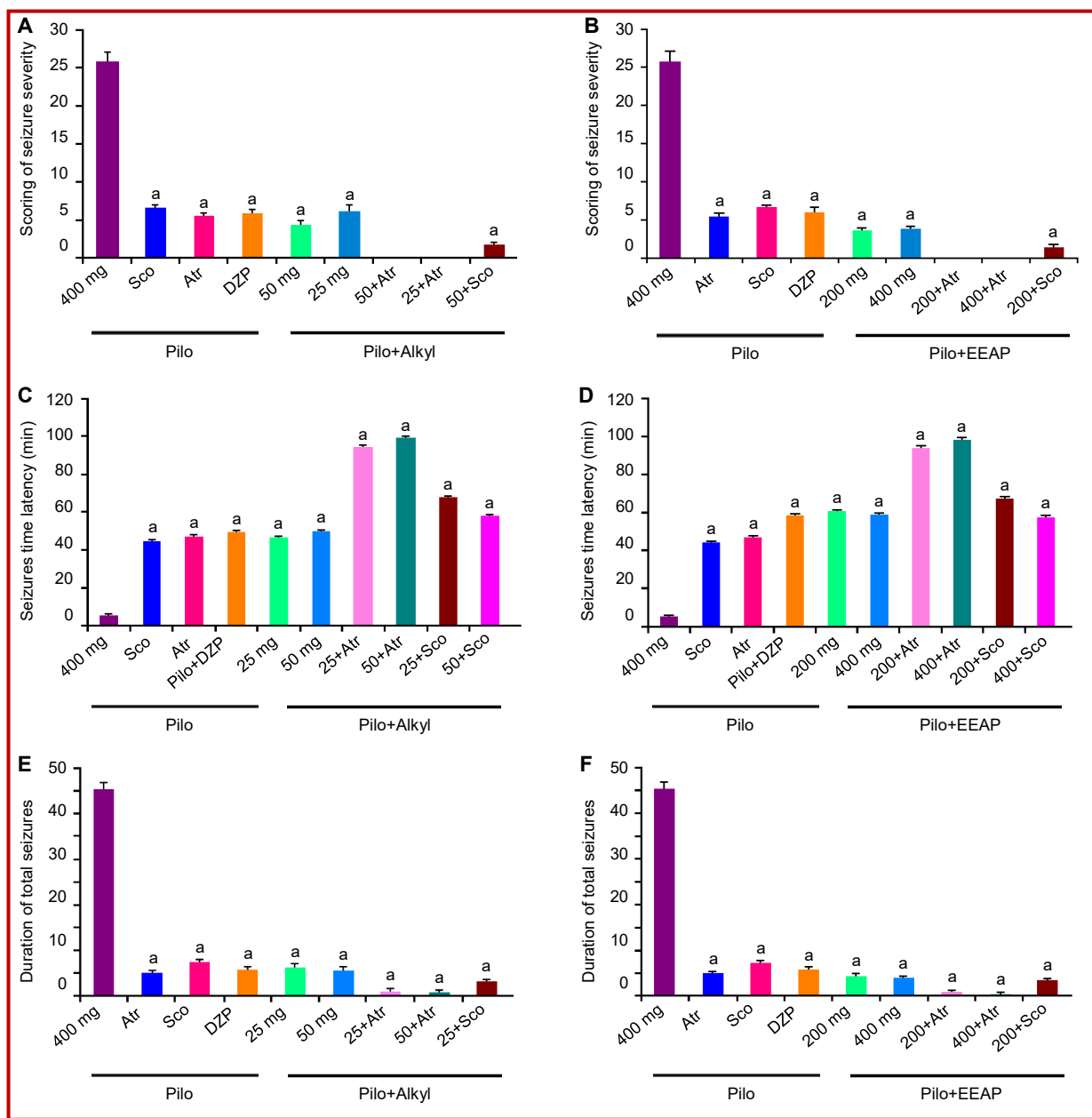


Figure 1: Total seizure scores (A,B), seizures time latency (C,D) and Duration of total seizures (E,F) of all the groups. Effect of treatment with EEAP (200, 400 mg/kg p.o), Alky (25, 50 mg/kg), (alkyl+scopolamine), (EEAP+ scopolamine), (alkyl+atropine), (EEAP+atropine) and, DZP on the pilocarpine induced convulsions. Results are expressed as means \pm S.E.M.. (n = 6 per group).

Percentage protection

Anticonvulsant studies with the extract showed significant protection in pilocarpine-induced convulsion models (data not shown).

Discussion

In the present study, root extracts of *A. pyrethrum* were assessed for their antiepileptic activity in the pilocarpine-based model of epilepsy in rats. The findings showed that the ethanol extract of *A. pyrethrum* and

alkylamides significantly reduced the duration of seizures and increased the latency period of pilocarpine-induced seizures in rats. These effects could be related to the extract effect on cholinergic receptors since the same effects were significantly reported using cholinergic receptor antagonists.

Pilocarpine is commonly considered as cholinomimetic drug widely used to induce convulsions in rodents. These seizures mimic the temporal lobe epilepsy in humans (Turski et al., 1989). The administration of a single dose of pilocarpine (400 mg) lead to induce status epilepticus in 83% of animals and mortality rate was

100% (Jope and Morrisett, 1986). The higher dose of pilocarpine produced motor limbic seizures more rapidly in the animals (Curia et al., 2008). In the pilocarpine model, which is a muscarinic agonist, the cholinergic receptor activation is involved in the onset of seizures (Turski et al., 1989). M₁ Muscarinic acetylcholine receptors (mAChRs) are implied in the pilocarpine induced seizures generation (Bymaster et al., 2003; Hamilton et al., 1997). Interestingly, Curia et al. (2008) noted that M₁ receptors knockout mice were incapable of developing epileptic seizures in response to pilocarpine. In this model, spontaneous seizure activity and neuronal loss are due to seizure-induced glutamate release (Clifford et al., 1987; Jope and Morrisett, 1986). Moreover, pilocarpine is well-known to induce seizures, can also cause pro-inflammatory action in the brain, the study by Marchi et al. (2007) suggests that pilocarpine caused acute peripheral pro-inflammatory changes that lead to a leakage of the blood-brain barrier before the occurrence of the epileptic state (Nicola, 2007).

In the current study, oral administration of ethanol extract of *A. pyrethrum* and alkylamides as well as atropine and scopolamine produced a significant anticonvulsant effect and reduced the seizure severity, increased the latency time of seizures, also note that, the duration of total seizures was higher in pilocarpine model compared to the treated groups with *A. pyrethrum*. Thus, the extract produced complete protection against pilocarpine model. In addition to anticonvulsant studies, *A. pyrethrum* extract was also screened for its acute and subchronic toxicity; it did not produce any behavioral abnormalities or mortality. Manouz et al. (2017) reported that the phytochemical screening of *A. pyrethrum* revealed the presence of flavonoids, alkaloids, terpenoids, tannins and saponins. The studies carried by Paramdeep et al. (2014) found out that alkaloids, flavonoids, terpenoids, saponins, and coumarins enhance gamma amino butyric acid (GABA) transmission. On the other hand, the co-administered of ethanol extract of *A. pyrethrum* and alkylamides with atropine which is an antagonist of muscarinic receptors has completely abolished the pilocarpine-induced seizures, this may suggest the cholinergic involvement in the actions of ethanol extract of *A. pyrethrum* and alkylamides. In addition, Jo et al., (2002) showed that, at the cellular level, stimulation of mAChRs and nicotinic acetylcholine receptor (nAChRs) on lateral hypothalamic (LH) neurons decreased and increased GABA release, respectively. This may lead us to suggest that, the blockade of muscarinic receptors by atropine as well as scopolamine could induce an increase of GABA.

Several studies have been focused on the central nervous system activities of *A. pyrethrum* extracts. The anticonvulsant effect was demonstrated against maximal electroshock induced convulsions in mice (Iswarya Devi, 2013). Fard and Shojaii, (2013) had also found that the *A. pyrethrum* extract in mice showed anticonvulsant

and myorelaxation activities. Earlier studies suggest that maximal electroshock and pentylenetetrazol might maintain their convulsive effects by inhibiting the activity of gamma amino butyric acid at GABA-A receptors (De Sarro et al., 1999; Vijayalakshmi et al., 2011). Pahuja et al. (2012) and Zaidi et al. (2009) had shown that *A. pyrethrum* extract has anti-epileptogenic effects on maximal electroshock and pentylenetetrazol models. This result is in agreement with the previously reported findings. According to these data and the present findings, it can be suggested that the anticonvulsant effect of the plant is probably mediated by different mechanisms or different molecules which may act individually or in synergy.

On the other hand, it is well established, that during single prolonged seizures or the induction of recurrent short seizures by chemoconvulsants or electrical stimulation in adult rats and mice, cause the rapid release of inflammatory mediators in areas of the brain where convulsive activity begins and spreads (Aronica et al., 2007; Fabene et al., 2010; Ravizza et al., 2010; Vezzani et al., 2000).

There have also been reports that during seizures, cyclooxygenase-2 (COX-2) is rapidly mediated in the main neurons of the forebrain, including hippocampal pyramidal cells and dentate granulate cells (Ciceri et al., 2002; Marcheselli and Bazan, 1996; Sandhya et al., 1998; Serrano et al., 2011; Takemiya et al., 2006; Yamagata et al., 1993). Furthermore, chemical analysis of the roots of *A. pyrethrum* showed that they mainly accumulate alkylamides (Zaidi et al., 2013) with an N-isobutylamide alkaloid, called pellitorine as the major active constituent. Furthermore, Crombie (1954) has isolated dodeca-2E,4E-dienoic acid isobutylamide (pellitorine) from *A. pyrethrum* and Cech et al., (2010) have noted that the identical root component which is pellitorine of *Echinacea purpurea* was responsible for immunomodulatory effects. As a result, plasma protein levels of certain pro-inflammatory cytokines (IL-8 and IL-6) will decrease, as well the expression of anti-inflammatory molecules such as IL-10. Interestingly, the alkylamides from *A. pyrethrum* extracts act as an inhibitor of cyclooxygenase (COX) and 5-lipoxygenase (LOX) enzymes (Rimbau et al., 1999). Based on all these data and current findings, it can be suggested that *A. pyrethrum* could act as antiepileptogenic by inhibiting of COX-2 and its ability to decrease certain pro-inflammatory as well.

Conclusion

A. pyrethrum possesses significant anticonvulsant activity which is abolished by atropine, and thus suggests that the possible mechanism may be mediated through cholinergic pathway. Furthermore, the anticonvulsant action of *A. pyrethrum* is due to its anti-

inflammatory properties.

Financial Support

Self-funded

Ethical Issue

In this study, all animal procedures were in strict accordance with the guidelines of the European Council Directive (EU2010/63). Care was taken to minimize the number of animals used for the experiments. All efforts have been made to limit animal suffering. All efforts were made to minimize any animal suffering, and the study met the ethical standards and approvals of the Council Committee of the research laboratories of the Faculty of Sciences, Cadi Ayyad University of Marrakech.

The acute and subchronic toxicity study was conducted according to the Organization for Economic Cooperation and Development (OECD) guideline No. 423 (OECD, 2001), where the 5,000 mg/kg limit test dose was used.

Conflict of Interest

Authors declare no conflict of interest

Acknowledgement

We thank Mr. Abderrazak Regragui for the expert animal handling required in this study.

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