The anticonvulsant activity of Asplenium nidus L. (Polypodiaceae) methanolic crude leaf extract in chemically induced tonic-clonic convulsions on Swiss mice

Holy May B. Faral*, Ronalyn B. Macaraig, Princess Marian B. Mojares, Reina Jean D. Caramat, Donnabel D. Abando, Laurina M. Balangi, Sheryl C. Aguila, Omar A. Villalobos
Pharmacy Department, College of Allied Medical Professions, Lyceum of the Philippines University, Batangas City
**hmfaral@yahoo.com*

ABSTRACT: Epilepsy is a chronic non-communicable disorder of the brain that affects people of all ages. Approximately, 50 million people worldwide have epilepsy, making it one of the most common neurological diseases globally. Asplenium nidus L. is a member of family Polypodiaceae which is commonly known as "bird's nest fern." It is used in many traditional medicines such as antipyretic, estrogenic, spasmolytic and medicinally as depurative and sedative. The present investigation was designed to evaluate the anticonvulsant activity of the Asplenium nidus L. in pentylnetetrazole and isoniazid-induced convulsions on 30 male Swiss mice equally divided into five groups. After acute toxicity test, oral treatment with Asplenium nidus methanolic extract at varying doses of 500, 750 and 1000 mg/kg BW was given to test animals. The efficacy of the plant extract was compared with diazepam as the standard drug (5 mg/kg BW) and PNSS (10 ml/kg BW) as the control. The significant of differences between groups was determined using Kruskal Wallis Test followed by the Mann Whitney $p < 0.05$. The data was presented as mean ± SEM in tables. Data were analysed using SPSS v.21 at 95% level of confidence. The three doses of A. nidus; 500 mg/kg BW, 750 mg/kg BW and 1000 mg/kg BW exhibited a decrease in the time of onset and duration of convulsion when compared to the group treated with normal saline ($p<0.05$) and showed no significant difference when compared to diazepam 5mg/kg BW ($p>0.05$). Results of study revealed that the methanolic crude leaf extract of the leaves of Asplenium nidus L. possess anticonvulsant effects by delaying the onset and decreasing the duration of convulsions.

Keywords: Epilepsy, Asplenium nidus, anticonvulsant, acute toxicity test

INTRODUCTION

According to World Health Organization (2016), there are approximately 50 million people worldwide who suffer from epilepsy, thus, making it one of the most common neurological diseases globally. It is a chronic non-communicable disorder of the brain that affects people of all ages. As cited from Chua (2007), epilepsy continues to be one of the leading causes of neurological consultations and admissions in the Philippines which affect 750,000 people.

Epilepsy is not a single entity but an assortment of different seizure types and syndromes originating from several mechanisms that have in common the sudden, excessive and synchronous discharge of cerebral neurons (Conway & Birbaum, 2015). Seizure results when a sudden imbalance occurs between the excitatory and inhibitory forces within the network of cortical neurons. Normal membrane conductance and inhibitory synaptic current breakdown and excess excitability spread either locally to produce a focal seizure or more widely to produce a generalized seizure (Mukhopadhyay, Kandar, Das, Ghos, & Gupta, 2012).

Epilepsy affects patients' cognitive functioning such as impaired attention or concentration, memory problems including forgetting things and short-term memory loss. Also, the emotional impact of having epilepsy was universally reported by children, adolescents, and adults. Types of emotional impact included anger, annoyance or frustration, particularly over limitations due to epilepsy, sadness, low mood, moodiness or depression, embarrassment, and worry or anxiety (Kerr, Nixon, & Angalakuditi, 2011).

Benzodiazepines are one of the most prescribed medicines for anxiety, insomnia, muscle relaxation, relief from spasticity caused by central nervous system pathology and epilepsy (Griffin III, Kay, Bueno, & Kaye, 2013). The same study stated that benzodiazepines cause a conformational change in the GABA-A receptor's chloride channel that hyperpolarizes the cell and enhances GABA's inhibitory effect throughout the central nervous system.

However, the concern about benzodiazepines causing dependence has led to recommendations that these drugs should be avoided (Tyrer & Baldwin, 2006). In an attempt to resolve this issue, interest has increased in alternative plant-related drugs. In recent years, it has become clear that flavonoids may play a role in enzyme and receptor systems of the brain, exerting various

effects on the brain by binding to the benzodiazepine site on the GABA-A receptor resulting in sedation, anxiolytic and anticonvulsive effects (Jager & Saaby, 2011).

Experimental evidences have clearly demonstrated that flavonoids exert antiepileptic activity by modulating the GABA-A-Cl¬-channel complex, as they are structurally similar to benzodiazepines. Thus, flavonoids may have a modulating role in the treatment of neurodegenerative diseases due to their phenolic nature, since they can disrupt cellular oxidative processes in the central nervous system (Diniz, et al., 2015). Several ethnomedicinal plants have been documented for the treatment of central nervous system disorders.

Pteridopytes are a group of plants commonly used as a source of medicine, food, ornamentals, as a source of fiber, bioremediation, and as organic fertilizer. Indeed, out of 1,100 species, only more than 50 species of these plants were reported to have medicinal values throughout the Philippines and 41 species which can be found in Mindanao (Amoroso V. B., Antesa, Buenavista, & Coritico, 2014).

Figure 1. A. nidus growing on a ground
Figure 2. A. nidus on a tree

Asplenium nidus L. is a member of family Polypodiaceae which is commonly known to the locals as “bird’s nest fern.” It forms large simple fronds visually similar to banana leaves, with the fronds growing to 50–150 centimeters (20–59 in) long and 10–20 centimeters (3.9–7.9 in) broad. They are light green, often crinkled, with a black midrib, and exhibit circinate vernation. A. nidus is native to east tropical Africa; temperate and tropical Asia (in Indonesia; East Timor; the prefecture of Kyushu, and the Ryukyu Islands of Japan; Malaysia; the Philippines; Taiwan; and Thailand); and in Australia (World Heritage Encyclopedia, 2001).

Several studies have documented for its traditional uses. Benjamin (2011) reported that the rootstock of A. nidus is used against fever and elephantiasis. It is used as emollient in coughs and diseases of the chest. Leaf is smoked to treat cold. Li (2008) stated that the whole plant extract is considered estrogenic, spasmolytic and treatment for debility, halitosis, and sores. Antibacterial activity of some ferns including A. nidus was also tested (Lai, Lim, & Tan, 2009). The phytochemical components namely, alkaloids, saponins, flavonoids, and tannins were present in Asplenium nidus extract (Amoroso V. B., Antesa, Buenavista, & Coritico, 2014). Certain kaempferol and quercetin flavonoids are the main phytoconstituents identified from the plant (Ling, Kian, & Hoon, 2009).

Kaempferol and Quercetin are polyphenolic flavonoid. It is a natural plant product with potentially useful pharmacological and nutraceutical activities. It is common in vegetables, fruits, plants and herbal medicines. Studies have shown that it reduces cancer, arteriosclerosis, cardiovascular disorder and serve as antioxidant and anti-inflammatory (Lau, 2008).

One study mentioned that Leea guineensis containing kaempferol and quercetin exhibited significant anticonvulsant activity in pentylnetetrazole induced seizures (Woode, Alagpulinsa, & Abotsi, 2011). Another research stated in Argyreia speciosa also contains kaempferol flavonoid that demonstrated an anticonvulsant effect on mice (Vyawahare & Bodhankar, 2009).

Several studies of anticonvulsant drugs used pentylnetetrazole and isoniazid to induce convulsions. Isoniazid is an effective and widely used drug in the treatment and tuberculosis. The administration of toxic amounts of isoniazid (INH) causes recurrent seizures, profound metabolic acidosis, coma and even death but the therapeutic dose of isoniazid rarely causes seizures (Puri, Kumar, Vishwakarma, & Behera, 2012).

In case a report, acute overdose of isoniazid results in an absolute pyridoxine (vitamin B6) deficiency. Pyridoxine is an essential cofactor in the synthesis of gamma amino butyric acid (GABA), which is the major inhibitory neurotransmitter in the central nervous system. The antituberculosis drug isoniazid reacts non-enzymatically with pyridoxal5'-phospate to form a metabolically inactive hydrazine, eventually interfering with GABA synthesis. Decreased levels of GABA cause a lowered seizure threshold (Okutur, Borlu, Yazici Ersoy, & Paksoy, 2006). One study stated that as pentylnetetrazole was given in mice at dose 40 mg/kg BW, it induced generalized seizure that acts for 1-2

hours. This seizure activity was manifested in two ways: (1) highly synchronous, large-amplitude activity in the thalamic and cortical field and (2) clonic jerking of the body and forelimbs (Fanselow, Reid, & Nicolelis, 2000).

However, this plant has still no direct scientific report for its neuropharmacological effect. Therefore, the researchers undertake the study to evaluate its anticonvulsant activity potential of the methanolic leaf axtract from A. nidus on pentylnetetrazole and isoniazid induced convulsions on male Swiss mice. Moreover, determination which among the dose of 500, 750 and 1000 mg/kg BW of the methanolic leaf extract of A.nidus produce a significant anticonvulsant effect by delaying the onset and decrease the duration of convulsion in male Swiss mice as compared to the standard drug and control were assessed. In addition to, the researcher determine the safety of the methanolic leaf extract of A.nidus at a maximum dose of 2000 mg/kg BW and to determine if the dose of the extract is dose-dependent.

This study hypothesized that the methanolic leaf extract of A.nidus will significantly produce an anticonvulsant effect on pentylnetetrazole and isoniazid induced convulsions on male Swiss mice. It is also expected that the anticonvulsant property of the A.nidus extract will be concentration dependent and demonstrate its anticonvulsant effect by delaying the onset and decreasing the duration of convulsions.

The findings of this study will benefit the society considering that epilepsy disorder is increasing worldwide today. The cumulative rate justifies the need for more effective and fewer side effects. This will also serve as a future reference for researchers who will pursue their study on this particular field. If the results are significant, this plant could serve as an possible source of compounds for medication that will be more readily accessible and inexpensive compared to the medications prescribed by most physicians.

The study covers the evaluation of anticonvulsant activity of the methanolic leaf extract of A.nidus on pentylnetetrazole and isoniazid induced convulsions on male Swiss mice. The researchers will use the experimental design composed of 30 young male Swiss mice with a weight 20-30 g for each chemical induced convulsion. The researchers will also conduct acute toxicity test to observe any behavioural changes such as scoring the severity of seizure is assess or grading the behavioural profile of the mice, the hyperactivity and reduced motor coordination and mortality for 24 hours after administration of the doses. Further

observation will be made until the 14th day. The experiment will not utilize any electronically induced convulsion. Furthermore, the study will not perform isolation of specific phytochemical constituent of A. nidus extract nor identification of active principle containing on the plant.

MATERIALS AND METHOD

Plant Collection and Authentication

The leaf part of Asplenium nidus was collected from Cuenca, Batangas last November 2016. The plant was identified and authenticated by the Bureau of Plant Industry by Manuel D. Ching, Chief CIPGR Section.

Preparation of the Plant Extract

About 1 kg plant material was collected and washed properly with water and dried for three weeks in a dark room. After washing, about 245g of dried powdered leaves was taken and macerated in 90% methanol at room temperature (25°C) over a period of 48 hours. Methanol containing the extract was then filtered. The extract was finally dried by rotary evaporator at 80 rpm, 40-50^{o}C. The percentage yield of Asplenium nidus methanolic leaf extract was computed based on the following formula:

$$\% \text{ yield} = \frac{WEB - WB}{WP} \times 100$$

WB – weight of the beaker
WEB – weight of the extract and beaker WP – weight of plant material

Drugs and Chemicals

Diazepam and Plain Normal Saline Solution (PNSS) was purchased from a local drugstore. Pentylnetetrazole, Isoniazid and Methanol were purchased from Sigma-Aldrich.

Animals and Diet

Male Swiss mice (1-3 months old) weighing 20-30 g was obtained from University of the Philippines-Los Baños, Laguna. The animals were housed and maintained in a room with a controlled temperature of 25 ± 1°C and lighting with light/dark 12:12-hour cycle in polypropylene or plastic cages. They were fed with balanced mice pellet diet and distilled water ad libitum.

Acute Toxicity Test

Acute toxicity of pure extract of A. nidus was evaluated according to the method described by Organization of Economic

Cooperation and Development Guideline 423 with slight modification (start dose at high level). The animals were fasted overnight by withholding food but not water. The methanolic leaf extract was administered orally at a dose of 2000mg/kg BW on three male Swiss mice that was initially separated into its respective groups and its mortality was observed for two days. No dose adjustment was made.

The mice were observed for its behavioural changes under the following parameters (Manjusha & Suneel, 2016):

Test	Directions	Scoring
Salivation	Wipe a filter paper beneath the jaw of the mouse. Observe for wetness.	(0)Filter paper remains dry. (1)Jaws and chin fur are observed to be wet. (2)Saliva actively drips down from the jaws.
Pilomotor erection	Observe contraction of pilomotor muscles, manifested by the erection of skin hair.	(0)Pilomotor erection disappears when touched upon. (1)Definite pilomotor is in the neck region. (2)Hair of entire mouse is erect and rough appearing.
Urination	Place a filtepaper of the cage. Notice changes in the consistency of the feces.	(0)Wet spots observed on the filter paper. (1) The upper part of the animal's hind legs is wet.
Diarrhea	Same procedure above. Notice changes in the consistency of feces.	(0)Soft consistency (1)Feces spread as it comes out from anus (2)Feces watery in nature
Circling motion	Observe if the mouse moves around the circles as if reaching for its tail.	(0)Absent (1)Present
Body Weight	Determine the body weight after the first day of administration of the drug at the end of the experiment/ before sacrificing the animal	(0)No change in body weight (1)Increase/decrease in body weight

After this, continuous observation was made until the 14th day after treatment (OECD, 2011). Furthermore, a gross necropsy was performed by a veterinarian for examination of pathologic lesions.

Induction of Convulsion

Pentylnetetrazole (PTZ) induced convulsion test was performed to evaluate the anticonvulsant property of drugs. Thirty male Swiss mice were divided into five groups; each group was comprised of six mice. The groups were designated as to the following:

Group 1: treated with PNSS (10mL/kg BW IP)
Group 2: treated with diazepam (5mg/kg BW IP)
Group 3: A. nidus extract (500 mg/kg BW po)
Group 4: A. nidus extract (750 mg/kg BW po)
Group 5: A. nidus extract (1000 mg/kg BW po)

Thirty minutes later, convulsions were induced by intraperitoneal (IP) administration of 60 mg/kg BW of PTZ. Following IP administration of PTZ, mice were placed in separate transparent glass cages (25 x 15 x 10 cm) and were observed for the occurrence of seizures over 30 minutes. The latency of convulsions (tonic convulsions), duration of tonic convulsions and mortality protection (percentage of deaths in 24 hours) was recorded.

For Isoniazid (INH) induced convulsion, thirty male Swiss mice was divided into five groups; each group was comprised of six mice. The groups were designated as to the following:

Group 1: treated with PNSS (10mL/kg BW IP)
Group 2: treated with diazepam (5mg/kg BW IP)
Group 3: A. nidus extract (500 mg/kg BW po)
Group 4: A. nidus extract (750 mg/kg BW po)
Group 5: A. nidus extract (1000 mg/kg BW po)

During the next 30 minutes, convulsions were induced by IP administration of 250 mg/kg BW of INH. After another 30 minutes, mice were placed in separate transparent glass cages (25 x 15 x 10 cm) and were observed and recorded for the occurrence of seizures over 30 minutes. The latency of convulsions (clonic convulsions), duration of tonic convulsions and mortality protection (percentage of deaths in 24 hours) was recorded.

The recorded experiment was sent to three evaluators. Double blind method was used in evaluation.

Statistical Analysis

Results of the experiments and observations were expressed as the mean ± standard error of the mean (SEM). The significant of differences between groups was determined using Kruskal Wallis Test followed by the Mann Whitney $P < 0.05$.

RESULTS

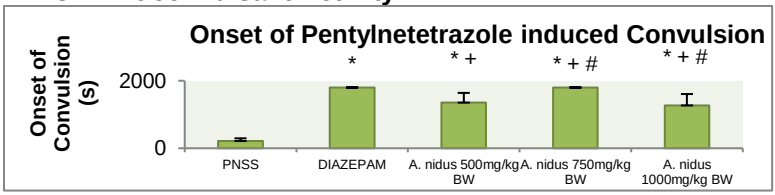
A. Plant Extract

About 16.5142 g of tea-like odor dark green crude extract was produced. The yield of A. nidus was found to be 6.74% w/w.

B. Acute toxicity test

The methanol extract of Asplenium nidus L. did not produce any mortality orally up to 2000mg/kg. There were no behavioural changes observed for 2 days up to 14 days. There were no visible signs of delayed toxicity and mortality observed when the animals were monitored for a further 14 days. The representative mice used in the study are apparently healthy as signified by the absence of gross pathologic lesions (haemorrhage, congestion, enteritis, pneumonia, atrophy, etc.) in the organs examined during necropsy.

C. Anticonvulsant Activity

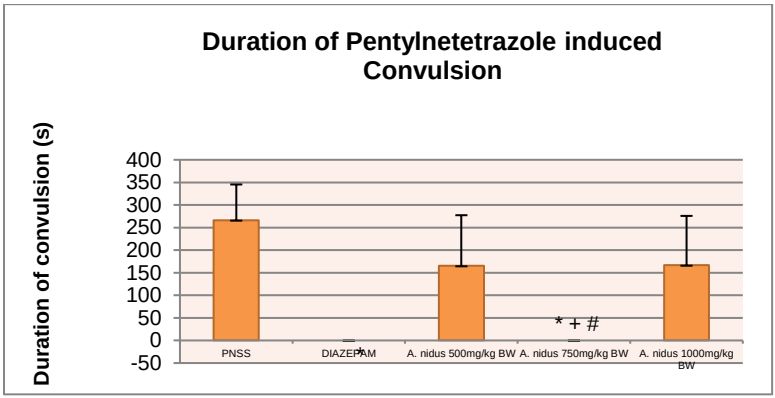


*vs. PNSS p-value <0.05; +vs. Diazepam p-value >0.05; #vs. A.nidus 500mg/kg BW p-value >0.05
Kruskal Wallis Test

Figure 1. Effect of methanolic extract of Asplenium nidus L. on onset of pentylnetetrazole induced seizure

Pentylenetetrazole was said to induce a tonic type of convulsion. Based from the data gathered and presented above (Figure 1), it showed that there is a significant differences in the onset of convulsion between groups (Kruskal-Wallis p-value <0.05). Mann-Whitney U test was used to statistically compare the differences among the group . Result showed that Diazepam (1800.00 ± 0.00) and the three doses of A. nidus; 500 mg/kg (1352.67 ± 288.21), 750 mg/kg (1800.00 ± 0.00) and 1000 mg/kg (1270.10 ± 336.12) exhibited a decrease in the time of onset of

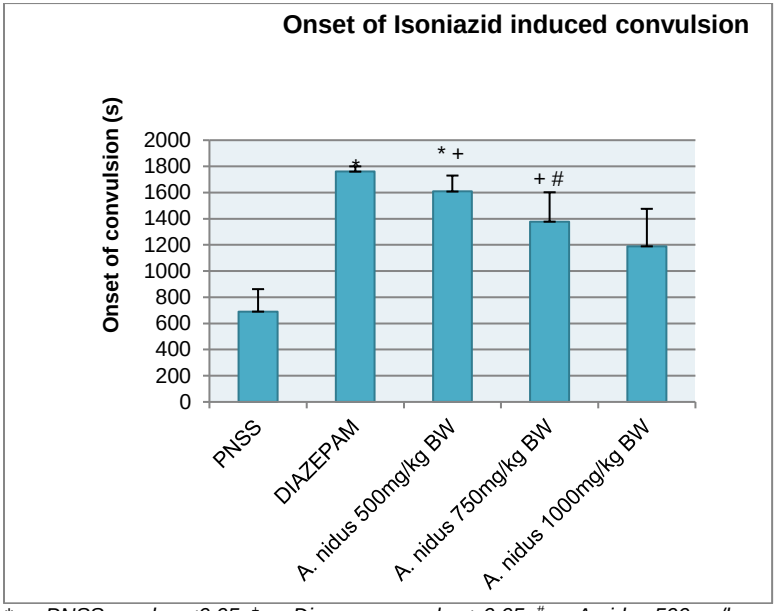
convulsion when compared to the group treated with normal saline (214.67 ± 79.48), this means that the extract and the three doses of A. nidus were able to delay the onset of convulsion produced by the PTZ (p-value <0.05). Three dose of A. nidus extracts were also statistically compared with the standard drug diazepam , it showed that the three extracts have shown no significant difference on delaying the onset of convulsion (p-value >0.05). Also, it is interesting to note that the 500 mg/kg BW extract of A. nidus showed comparable effect when compared to the two higher dose of A. nidus extract, 750 mg/kg and 1000 mg/kg at p-value >0.05.



**vs. PNSS p-value <0.05; +vs. Diazepam p-value >0.05; #vs. A.nidus 500mg/kg BW p-value >0.05 ; Kruskal Wallis Test*

Figure 2. Effect of methanolic extract of Asplenium nidus L. on duration on pentylnetetrazole induced seizure

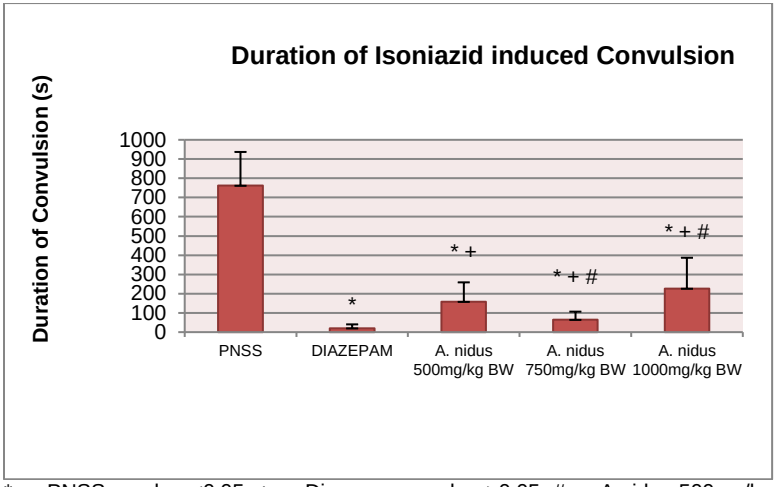
Figure 2 showed that there is a significant difference in the duration of convulsion between groups Kruskal- Wallis p-value <0.05). Mann-Witney U test was used to statistically compare the differences among the group. Result showed that Diazepam 5mg/kg BW (0.00 ± 0.00) and the three doses of A.nidus; 500 mg/kg BW (166.50 ± 109.31) exhibited decrease in the time of duration of convulsion produced by the PTZ (p-value < 0.05). Three dose of A.nidus extract were also statistically compared with the standard drug diazepam , it showed that only the 750mg/kg extract has shown no significant difference on decreasing the duration of convulsion (p-value >0.05).



**vs. PNSS p-value <0.05; +vs. Diazepam p-value >0.05; #vs. A.nidus 500mg/kg BW p-value >0.05 ; Kruskal Wallis Test*

Figure 3. Effect of methanolic extract of *Asplenium nidus* L. on onset of Isoniazid induced seizure

Isoniazid was said to induce a clonic type of convulsion. Based from the data gathered and presented above (Figure 3), it showed that there is a significant differences in the onset of convulsion between groups (Kruskal-Wallis p-value <0.05). Mann-Witney U test was used to statistically compare the differences among the group . Result showed that Diazepam (1760.50 ± 39.50) and the three doses of A.nidus; 500 mg/kg (1608.33 ± 121.24), 750 mg/kg (1378.17 ± 223.86) and 1000 mg/kg (1189.83 ± 285.65) exhibits decrease in the time of onset of convulsion when compared to the group treated with the normal saline (690.83 ± 171.07), this means that the extract and the three doses of A.nidus were able to delay the onset of convulsion produced by the INH (p-value <0.05).Three doses of A.nidus extract were also statistically compared with the standard drug diazepam , it showed that 500 mg/kg and 750 mg/kg extracts have shown no significant difference on delaying the onset of convulsion (p-value >0.05).



*vs. PNSS p-value <0.05; +vs. Diazepam p-value >0.05; #vs. A.nidus 500mg/kg BW p-value >0.05
Kruskal Wallis Test

Figure 4. Effect of methanolic extract of Asplenium nidus L. on duration of Isoniazid induced seizure

Figure 4 shows that there is a significant difference in the duration of convulsion between groups, (Kruskal-Wallis p-value <0.05). Mann-Witney U Test was used to statistically compare the differences among the group . Result showed that Diazepam (20.17 ± 20.17) and the three doses of A.nidus; 500 mg/kg (158.17 ± 100.66), 750 mg/kg (64.50 ± 41.99) and 1000 mg/kg (226.33 ± 160.35) exhibits decrease in the time of duration of convulsion when compared to the group treated with normal saline (614.67 ± 175.08), this means that the extract and the three doses of A.nidus were able to decrease the duration of convulsion produced by the INH (p-value <0.05). Three dose of A.nidus extract were also statistically compared with the standard drug Diazepam , it showed that the three extracts have shown no significant difference on decreasing the duration of convulsion (p-value >0.05).

DISCUSSION

The present study was proposed to assess CNS anticonvulsant effect of methanolic crude leaf extract of an ethnomedicinal plant, Asplenium nidus L.

Delaying the onset and decreasing the duration of seizures induced by pentylnetetrazole and isoniazid in laboratory animal is

the most common screening test used for evaluating potential anticonvulsant drugs. The results of the present study indicate that A.nidus extract possesses anticonvulsant activity. The present study showed the anticonvulsant effects of the plant extract in both pentylnetetrazole and isoniazid induced convulsion by delaying the onset and decreasing the duration of convulsion.

The effect of most anticonvulsant agents acts by enhancing the inhibitory neurotransmitter, GABA. It is well-established that PTZ and INH induced convulsions were produced due to GABA receptor and level modification respectively in the CNS. These findings supported (Diniz, et al., 2015) that flavonoids exert antiepileptic activity by modulating the GABA-A-Cl¬-channel complex, as they are structurally similar to benzodiazepines. Thus, flavonoids may have a modulating role in the treatment of neurodegenerative diseases due to their phenolic nature, since they can disrupt cellular oxidative processes in the central nervous system.

Therefore, A. nidus extract might possibly be producing an anticonvulsant action by counteracting oxidative stress and antagonizing conformational change of GABA that occurs during convulsion. This effect agreed with the pharmacological effect of BZDs in anticonvulsant activity.

SUMMARY

It is determined that the methanolic leaf extract of A.nidus at a maximum dose of 2000 mg/kg BW is safe and does not produced any signs of toxicity and abnormal behaviour changes up to 14 days. Asplenium nidus L. methanolic crude extract demonstrated an effective and significant anticonvulsant activity by delaying the onset and decrease the duration of convulsion in male Swiss mice as compared to the control both on PTZ and INH induced convulsions. The relationship of the dose of the extract to the activity does not display a dose-dependent relationship.

CONCLUSION

Results of the present study revealed that the methanolic crude leaf extract of the leaves of Asplenium nidus L. possess anticonvulsant effects by delaying the onset and decreasing the duration of convulsions. This plant and its flavonoids might serve as lead compounds for the synthesis of drugs which could be used in the management of these neurological disorders. The relationship of the dose of the extract to the activity does not display a dose-dependent relationship.

RECOMMENDATIONS

Based on the results and conclusions, the researchers would like to recommend a GABA test to confirm the crude extracts activity on the brain. Other parts of the A. nidus plant such as the roots and stem can also be tested for the determination of their anticonvulsant properties since this study only used leaves.

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