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PRELIMINARY PHYTOCHEMICAL, GC-MS ANALYSIS AND ANTIEPILEPTIC STUDIES ON THE METHANOL LEAF EXTRACT *OF BIXA ORELLANA* (BIXACEAE)

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ABSTRACT

Background and aim: Epilepsy is a public health challenge, affecting up to 50 million people worldwide. In sub-Saharan Africa alone, 5.4 million people mainly 20-49 years are affected. Adverse effects and high cost limit use of conventional therapies. The aim of this study was to evaluate the anti-epileptic properties of *Bixa orellana* and chemically characterize its constituents.

Methods: Twenty-five mice were divided into five groups (n = 5). Negative Control group I received 10 ml/kg tween 80 in addition to food and water *ad libitum*. Groups II, III and IV received oral doses of leaf methanol extract (200, 400 and 800 mg/kg, respectively) plus Isoniazid. Positive control group V received Sodium valproate (30 mg/kg b.w. per oral) plus Isoniazid. Animals were observed for myoclonic seizures, latency to convulsion and death. Phytochemical analyses and gas chromatography-mass spectroscopy analysis were carried out.

Results: *B. orellana* significantly ($p < 0.001$) increased mean onset of seizure in all treatment groups compared to negative control. Mean onset for control (3.35±1.00 minutes) was incomparable to extract-treated groups (19.00±1.22, 20.25±4.27, and 20.00±2.54 minutes), respectively. GC/MS analysis showed the presence of aliphatic compounds (35.16%), ester (21.50%), aldehydes (14.97%), fatty acids (13.61%) and polyphenolics (9.33%) which accounted for 95% of the compounds.

Conclusion: *B. orellana* increased the latency to onset of Isoniazid induced seizure and this effect is believed to be due to the presence of secondary metabolites like alkaloids, tannins, flavonoids, and saponins present.

Key words: *Bixa orellana*, isoniazid, antiepileptic activity, GC/MS

INTRODUCTION

Epilepsy could be referred to as a neurological disorder in which the brain nerves are disturbed leading to remodeling of membrane phospholipids [1]. There are short episodes of abnormal brain electrical activity and is known to affect approximately 50 million persons worldwide. This makes it the third most common brain disorder with 30 percent refractory rate [2,3]. Abnormal increase in the stimulation of N-methyl-D-aspartate (NMDA) receptors and lack of inhibitory gamma-aminobutyric acid A ($GABA_A$) neurons leading to increase in intracellular Ca^{2+} and excitotoxicity is believed to be at the foundation of seizures [1]. There is a school of thought that believes that oxidative stress could be a factor in the initiation and progression of seizures [4].

Conventional treatment of seizures involves a range of drugs usually referred to as antiepileptic drugs (AEDs). Among these AEDs are drugs like Valproate and Ethosuximide which are the main stay in the management of generalized seizure. AEDs though effective, are known to be associated with side effects, resistance, poor safety profile and high cost which is making alternative medicines to be in higher demand [5]. The high cost of AEDs especially in sub–Saharan Africa is a disadvantage as more than 50% of the population live on less than $2/day. There is major need for the development of alternative therapies that will not only be affordable but effective in modifying and preventing epileptic convulsions [6]

Use of plants in the treatment and management of diseases is not new. Evidence abound in the literature on the use of different plants in the management of epilepsy and convulsive disorders [7]. *Bixa orellana,* also known as annatto, is a perennial shrub that grows in different parts of the globe. It is claimed to possess different properties including anti-infective, antidiabetics, antioxidant, analgesic, antidiarrheal, antiulcer for the management of neurological problems, and snake bites [8]. The number of diseases that can be managed using *B. orellana* is so numerous that Rivera-Madrid, Aguilar-Espinosa [9] posited that the healing attributes of *B. orellana* has not all been studied especially its antiepileptic property. These researchers believed that the potential as a source of new drugs to aid modern day scientific discovery of drugs is enormous.

The aim of this study was to characterize the leaf extract of *B. orellana* using GC/MS and also evaluate its anti-seizure property using established animal model.

MATERIAL AND METHODS

Collection and identification of plant material: Fresh leaves of *B. orellana* were collected from a forest at the back of University of Nigeria, Nsukka, Enugu State. The plant was identified and authenticated by a taxonomist, Mr. Ozioko working with Medicinal Plant Department of Biodiversity Development and Conservation (BDPC INTERCCED) Program where a specimen voucher sample with specimen number INTERCEED 34512 BIXA was deposited in their Herbarium. The fresh leaves were cleaned and air dried away from direct sunlight. The leaves were ground into fine powder using a clean mechanical grinder and stored in a clean dry plastic container until use.

Drugs and Chemicals: Isoniazid (INH) was purchased from SigmaAldrich Inc., St. Louis, MO, USA. Sodium valproate, VPA (Epilim®, Sonofi-Synthelabo Ltd-UK) was purchased from a Pharmacy located at Enugu, Enugu state.

Extraction: Dried plant material (100 g) was macerated with 70% methanol for 48 hours

with occasional shaking. Resulting mixture was filtered using muslin cloth followed by Whatman filter paper (No. 1). The filtrate obtained was reduced to 10% of its initial volume using rotary evaporator and dried to a constant mass on water bath at less than 40°C and stored at 4°C until use [10].

Phytochemical screening of extract

Phytochemical screening was performed to ascertain the different classes of phytochemicals (alkaloids, saponins, tannins, flavonoids, steroids, terpenoids, glycosides, protein, and carbohydrate) present based on standard method [11]. Quantitative screening for alkaloids, tannins, flavonoids, and saponins was performed as described by Ezeonu and Ejikeme [12].

Gas chromatography-mass spectroscopy analysis

The gas chromatography-mass spectroscopy (GC-MS) analysis was carried out using GC-MS-Buck M910 gas chromatograph coupled to a mass detector. The GC was equipped with a capillary column coated with VF-5 (30 m + 10 m EZ guard column x 0.25 mm internal diameter, 0.25 μm film thickness). GC condition comprised injector and detector temperatures (250 ºC and 280ºC, respectively), oven temperature programmed as follows: 120 ºC held for 4 min, ramp at 10 ºC/ min to 180 ºC, held for 2 min, and finally ramp at 5 ºC/ min to 300 ºC. Helium was used as carrier gas at a flow rate of 1.0 mL/ min and detector make-up gas of 29 mL /min. The injection volume of the GC was 10.0 μL. Identification of the compounds was based on spectra which were compared with the database spectrum of known components stored in the GC-MS library using National Industrial Security Program (NISP) search. Measurement of the peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

Animal handling: Twenty-five male Swiss Albino mice (8 weeks old, 18-25 g) were obtained from the animal house of the Department of Pharmacology, Enugu State University of Science and Technology, Enugu State Nigeria. Animals were allowed to acclimatize under standard conditions for two weeks prior to start of the experiment. Within the period, the animals had free access to chaw and water *ad libitum* and were maintained on a 12-h light-dark cycle. All the experimental studies were reviewed and approved by the Enugu State University of Science and Technology Ethical Committee (ESUT/PHARM/15/040). The approved experimental conditions adhered to the guidelines for the care and use of animals in research of the Enugu State University of Science and Technology.

Acute toxicity studies: Different concentrations *B. orellana* methanol extract (10, 100 and 1000 mg/kg per body weight in the first phase and 1600, 2900 and 5000 mg/kg body weight in the second phase) were administered orally to the mice as described by Lorke [13]. Animals were observed daily for toxicological manifestations like feeding pattern changes, behavioral changes, neural and autonomic pathologies. There were no feeding and behavioral changes nor mortality recorded during this period even up to the dose of 5000 mg/kg [14]

Seizure Induction: Isoniazid (INH) powder was dissolved in distilled water to obtain a stock solution of 60 mg/ml [15]. Mice were treated with 300 mg/kg Isoniazid i.p., and were observed for one hour, noting the time interval for the onset of the seizure activity, the pattern of seizure development and death.

Experimental Design: The animals were randomly divided into five groups 1-V with each group consisting of five animals. They were treated with either the standards or extract at different doses one hour before the administration of Isoniazid solution. Group I

received 10 ml/kg of Tween 80 in addition to food and water *ad libitum*. Group II–IV received 200, 400 and 800 mg/kg extract, respectively per oral plus Isoniazid. Group V received Sodium valproate (30 mg/kg b.w. per oral) plus Isoniazid. Thereafter, animals were observed for two hours for the onset of myoclonic seizures, latency to convulsion and death.

Statistical Analysis: Data obtained were analyzed using Graphpad prism 9 (www.graphpad.com, San Diego California). The data were analyzed based on one-way analysis of variance and subjected to Duncan´s post hoc test. P values of 0.05 or less were considered statistically significant and the results presented as mean ± standard deviation.

RESULTS

Phytochemical Screening:

Leaf methanol extract of *B. orellana* was found to be rich in alkaloids, tannins, proteins, terpenoids, flavonoids, and steroid (Table 1). Yield of alkaloids, tannins, flavonoids and saponins in 2 g extract is presented in Table 2.

Table 1: Qualitative phytochemical analysis of *Bixa. orellana*

Phyto-constituents	Inference
Alkaloids	+++
Saponins	+
Tannins	+++
Flavonoids	++
Steroids	++
Terpenoids	++
Glycosides	+

Key: (+) = slightly present, (++) = moderately present, (+++) = highly present

Table 2: Quantitative phytochemical analysis of *Bixa orellana*

Phytoconstituents	Percentage yield (% w/w)
Alkaloids	7.8
Tannins	6.4
Flavonoids	4.8
Saponins	3.4

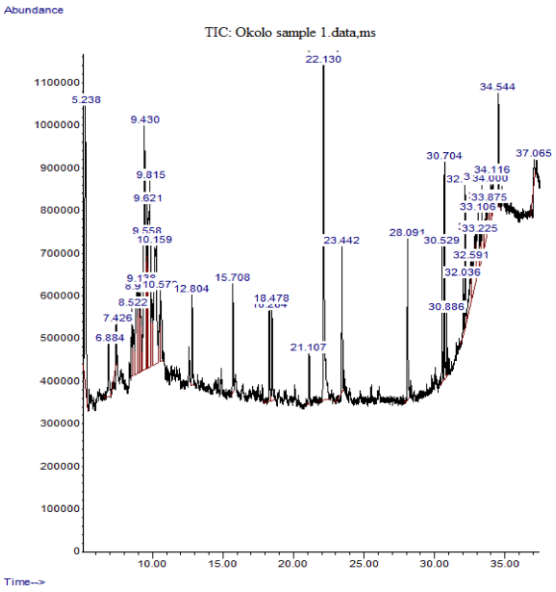

Figure 1. Gas chromatography-mass spectrometry chromatogram of methanol extract of *Bixa orellana* leaf.

Table 3: Gas Chromatographic-Mass spectrometric analysis of *Bixa orellana*

Peak No	R_t	% Area	Molecular Formula/ Molecular weight (g/mol)	Compound
1	5.238	8.22	$C_{18}H_{32}O$ (264.40)	9,12-Octadecadienal
2	6.884	2.43	$C_{16}H_{34}$ (226.44)	n-Hexadecane

3	7.426	0.44	$C_6H_4Cl_2$ (147.00)	1,4-dichlorobenzene
4	8.522	1.45	$C_{25}H_{50}O_3$ (398.70)	Carbonic acid, decyl tetradecyl ester
5	8.878	3.17	$C_{35}H_{70}$ (490.90)	Pentatriacontan-17-ene
6	8.985	3.29	$C_{11}H_{24}$ (156.31)	Undecane
7	9.138	1.97	$C_{24}H_{50}$ (338.70)	Tetracosane
8	9.430	9.38	$C_{14}H_{26}O_3$ (242.35)	Tetradecylcarbonate
9	9.558	1.87	$C_{35}H_{70}$ (490.90)	Pentatriacontan-17-ene
10	9.621	3.37	C_9H_{20} (128.25)	2,6-dimethyl-heptane
11	9.815	8.69	$C_{13}H_{24}O_3$ (228.33)	Nonylprop-1-ene-carbonate
12	10.159	8.23	$C_{12}H_{26}$ (170.33)	5-Methylundecane
13	10.579	2.43	$C_{10}H_{23}NO$ (173.30)	O-decylhydroxylamine
14	12.804	1.56	$C_{14}H_{29}Cl$ (232.83)	1-chlorotetradecane
15	15.708	1.79	$C_{13}H_{27}Cl$ (218.80)	1-chlorotridecane
16	18.284	1.79	$C_{20}H_{40}$ (280.50)	1,2-Diethylcyclohexadecane
17	18.478	1.98	$C_{18}H_{34}O_2$ (282.50)	Ethenyl hexadecanoate
18	21.107	0.93	$C_{16}H_{34}O$ (242.44)	2-Hexyldecan-1-ol
19	22.130	9.33	$C_{14}H_{22}O$ (206.32)	2,4-Di-tert-butylphenol
20	23.442	2.67	$C_{22}H_{42}O_2$ (338.60)	(Z)-Docos-13-enoic acid
21	28.091	4.77	$C_{19}H_{38}$ (266.50)	Nonadec-1-ene
22	30.52	0.60	$C_{14}H_{10}O_5$ (258.23)	3-POenoxyphthalic acid
23	30.704	1.54	$C_{20}H_{40}$ (280.50)	(E)-Icos-3-ene
24	30.886	0.44	$C_8H_{16}O_3$(160.21)	2,2,5-Trimethyl-1,3-dioxan-5-yl)methanol
25	32.036	0.37	$C_{18}H_{34}O_2$ (282.50)	(Z)-octadec-9-enoic acid
26	32.181	1.17	$C_{22}H_{44}$ (308.60)	Docos-1-ene
27	32.591	2.15	$C_{18}H_{34}O$ (266.50)	(E)-Octadec-9-enal
28	32.934	3.31	$C_{18}H_{34}O_2$ (282.50)	9-Octadecenal
29	33.106	2.26	$C_{18}H_{34}O_2$ (282.50)	(Z)-octadec-9-enoic acid

30	33.225	0.34	$C_{18}H_{34}O$ (266.50)	(Z)-octadec-13-enal
31	33.375	2.51	$C_{18}H_{34}O_2$ (282.50)	(Z)-octadec-11-enoic acid
32	33.679	1.71	$C_{32}H_{62}O_2$ (478.80)	Tetradecyl octadec-9-enoate
33	33.875	0.98	$C_{21}H_{40}O_4$ (356.50)	2,3-dihydroxypropyl octadec-9-enoate
34	34.000	0.95	$C_{14}H_{26}O$ (210.36)	(Z)-Tetradec-7-enal
35	34.116	1.42	$C_{18}H_{34}O_2$ (282.50)	(E)-octadec-13-enoic acid
36	34.544	1.44	$C_{18}H_{34}O_2$ (282.50)	Octadec-11-enoic acid
37	37.065	0.25	$C_{18}H_{34}O_2$ (282.46)	(13E)-13-Octadecenoic acid

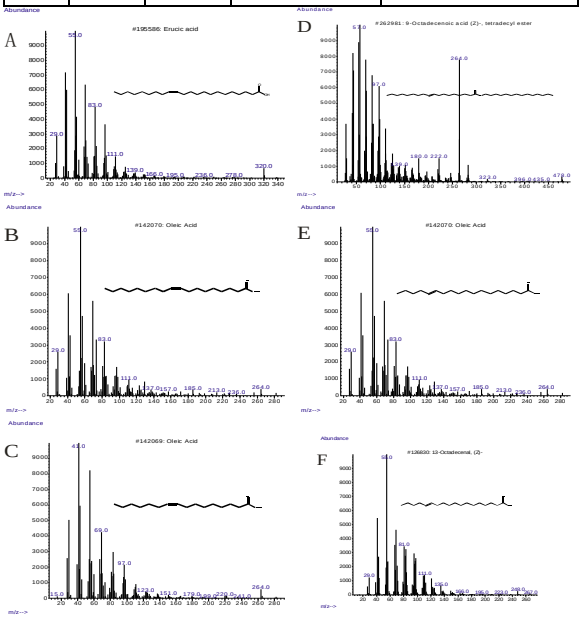

Figure 2. Mass spectra of fatty acids found in the extract of *Bixa orellana*: (A) Erucic acid, (B) Oleic acid, (C) Cis-Vacenic acid, (D) Tetradecyl octadec- 9-enoate, (E) (E)-Octadec–13-enoic acid, (F) Trans-13-octadecenoic acid

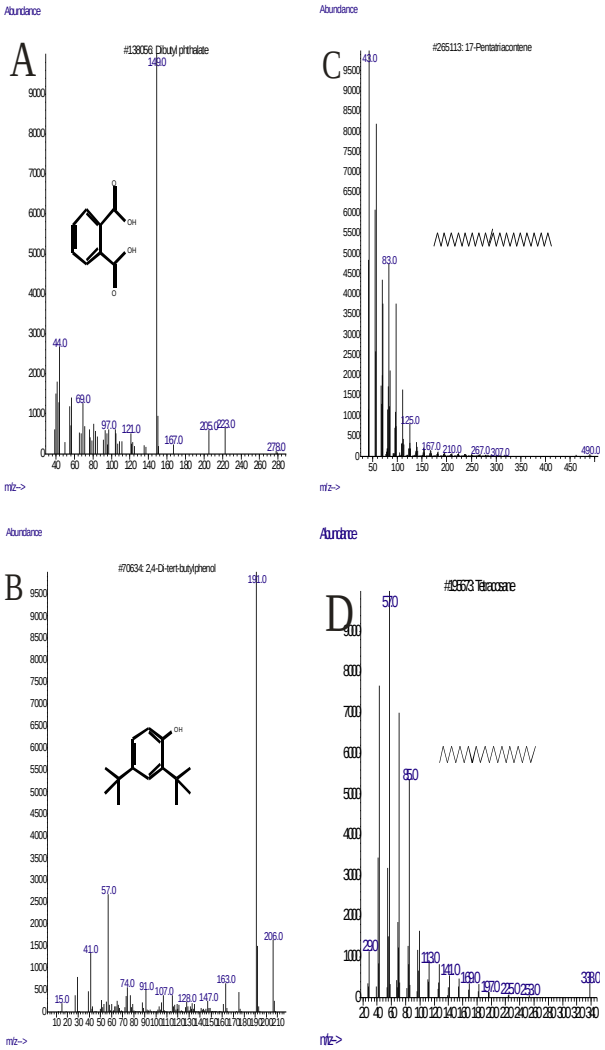

Figure 3. Mass spectra of compounds with putative properties *B. orellana* extract: (A) 1, 2-Benzenedicarboxylic acid, (B) 2, 4- di-tert-butylphenol, (C) 17-Pentatriacontene, (D) Tetracosane

Effect of methanol extract of *B. orellana* on isoniazid-induced seizures

Exposure of animals to Isoniazid induced tonic-clonic seizures in all experimental groups. Treatment with different doses of *B. orellana* and standard antiepileptic drug (200 mg/kg valproic acid) significantly increased

($p < 0.0001$) latency of onset of seizure, but not of seizure itself in all treatment groups (Table 4).

DISCUSSION

Qualitative phytochemical analysis of extract showed that it is rich in alkaloids, tannins, flavonids, steroids and terpenoids (Table 1) while the quantitative analysis gave the percentage quantities present (Table 2). Tannins and flavonoids are well known for their antioxidant properties which may combat oxidative stress that had been implicated in epileptic convulsion [2,4]. This may probably be the reason why Rivera-Madrid, Aguilar-Espinosa [9] concluded that modern science have not studied all the healing attributes of *B. orellana*. Several studies had established the anticonvulsant properties of alkaloids. Mishra *et al*. [16], using *in vitro, in vivo* and *in silico* techniques showed that an alkaloid like hexahydropyridine, a cyclohexane exerted anticonvulsant properties by possibly antagonizing sodium and calcium channels, and also by enhancing GABA signalling pathway. Furthermore, an alkaloid-rich fraction of *Jatropha gossypiifolia* exhibited marked anticonvulsant effect by reducing the frequency and duration of convulsions using a similar model. It is scientifically reasonable to believe that the increase time for onset of conlsion in *B. orellana*-treated mice could be related to the alkaloid content [17]. Also, the presence of flavonoids and tannins could offer an explanation towards the observed anticonvulsant property of *B. orellana.*

Both tannins and flavonoids are known antioxidants [18], and since the convulsant property of Isoniazide involves generation of oxidative stress, ameliorative property exhibited by *B. orellana* could be linked to the antioxidation [19,20]. Steroids, terpenoids and proetins had been associated with anticonvulsive actitivites [21,22].

Evidence from contemporary researches attributed the convulsive potential of Isoniazid to lack of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) and abnormal increase in the stimulation of N-methyl-D-aspartate (NMDA) receptors in the brain circuitory controling motor activity [1]. Use of Isoniazid, a chemoconvulsant in screening of compounds for its antiepileptic property is not new and has universal acceptance [23]. Badrinath and John [24] opined that Isoniazide metabolism produces two major compounds; hydrazine and monomethylhydrazine which are believed to be directly involved in the mileu of toxicological manifestations affecting the neurocircuitory. From the results obtained in this study, administration of Isoniazid resulted in hind-limb, tonic-clonic convulsion (Table 4) with associated mortality in mice, which is in agreement with similar works [25]. Extract-treated groups had delayed onset of tonic clonic convulsion, but not of protection one hour post Isoniazide administration. Valproic acid-treated goup had complete protection both from tonic clonic seizure and death after one hour of Isoniazide administration. Scientifically, management of seizures involves pharmaceutical armemetarium that modulates membrane ion channels or GABAergic or glutamatergic transmission [1].

Treatment of seizure induced animals with *B. orellana* extract significantly ($p < 0.0001$) increased latency of onset, but could not decrease that of death. While most researchers working with herbal products recorded positive effects in both onset and mortality [25,26], this present research only observed positive result in onset. The

increase in latency of onset is believed to be based on the effect of *B. orellana* on $GABA_A$ receptors. It is known that *B. orellana* has the ability to down regulate protein kinase C (PKC) and Nitric oxide cyclic GMP signalling cascade [27]. PKC is widely distributed in the brain where it phosporylates $GABA_A$ receptors [28]. Inhibition of PKC is known to partly account for the mechanism of action of some conventional antiepileptic drugs like valproic acid [29]. It is also known that $GABA_A$ receptors function could be regulated by lipid bilayer elasticity and that poly unsaturated fatty acids (PUFA) could regulate membrane protein function via this elasticity [30]. *B. orellana* is richly endowed with PUFA (Table 3 and Figure 2) which could partly account for the activity since PUFA's are known to delay onset of convulsion in animal models [31]. PUFAs also dampen excitability by opening neuronal M-channels [32]. The biological effect of suppresing NO-cGMP is also known to involve control of seizure threshold via glutamatergic and GABAergic pathways since known inhibitors of neuronal NO synthase like 7- nitroindazole is known to have anti-convulsant effect [33,34]. Conventional antiepileptic drugs like valproate and ethosuximide are known to be inhibitors of NO generation [35]. It is therefore scientifically reasonable to believe that the effects of *B. orellana* on $GABA_A$ and NO could partly account for this increase in latency of onset.

Furthermore, Isoniazid is known to cause increase in oxidative stress as part of its mechanism of induction of convulsion [36]. Natural herbs rich in antioxidants may attenuate isoniazid-induced epileptogenic convulsion. *B. orellana* is suspected to contain antioxidants such as phenolics, tannins and flavonoids molecules like 1, 2 – benzenedicarboxylic acid (Table 3 and Figure 3) which had been shown to be effective in stress-induced neurotoxicity [37,38].

The antioxidant compound, 2, 4-di-tert-butylphenol, found in *B. orellana* could increase seizure onset time with modulation of tonic-clonic. Zhu *et al*. [2] believed that the anticonvulsant activity of antioxidants could be attributed to alteration in levels of endogenous enzymes that may be key to convulsion, inhibition of oxidative injury and or binding to benzodiazepine site of $GABA_A$ receptor resulting in reconstitution of GABA pool [1]. Oleic acid, a monounsaturated omega 9 fatty acid that reversibly open blood brain barrier is believed to decrease excitability due to shift in hyperpolarizing direction of the inactivation curves of both sodium and calcium ion currents causing slowed recovery from inactivation analogous to its effect in arryhtmias [39]. Banderó *et al*. [40] and Rhoads *et al*. [41] believed that the anticonvulsant property of oleic acid could possibly increase latency of onset based on modulation of neuroactive amino acid transport in nerve endings.

There exists an intricate relationship between epilepsy and inflammation with both being a positive feedback loop. Inflammatory mediators are released from brain cells and peripheral immune cells following convulsion and inturn help to trigger further convulsions [3]. Many herbs contain metabolites that can terminate these cascade of inflammatory process thereby terminating further reaction. 2, 4-Ditert-butylphenol, 17 -pentatriacontene and oleic acid found in *B. orellana* are known to possess anti-inflammatory property [9,42,43] that could terminate inflammatory mediators, thereby extending latency of onset. Also worth mentioning here is the anti-inflammatory property of *B. orellana* that has been attributed to anti-bradykinin activity via inhibition of nitric oxide [44].

Involvement of peroxisome proliferator-activated receptors (PPAR's) in the pathegenesis of convulsion is also important. PPAR's are known to protect brain cells from many neurological diseases. Pioglitazone, a known agonist of PPAR protected rat brain from pentylenetrazole induced epilepsy, an action believed to have been exerted via rapamycin signalling pathway [45]. Erucic acid ((Z)-Docos-13-enoic acid), a monounsaturated omega 9 fatty acid component of *B. orellana* is a known nutritional ligand of PPAR [46]. Its effect is believed to stem from the accumulation of this fatty acid in tissues and body fluids as a result of slow degrading capacity [47]. PPAR's affect both incidence and threshold of seizure [48].

It is also a known fact scientifically that aggregation of β-amyloid protein lead to convulsion becouse it causes sustained depolarisation. Tetracosane, a natural component of *B. orellana* has the capacity to inhibit β-amyloid protein aggregation therby exerting a protective role that could manifest as delay on convulsive onset [49]. The potential of *B. orellana* to increase latency of unset but not mortality may be attributed to kinetics of the secondary metabolites to traverse the blood brain barrier and achieve a protective concentration.

CONCLUSION

Conclusively, our results showed that *B. orellana* leaf extract delayed the induction of seizure in Isoniazide induced model and may have a place in the development of more modern and effective therapy against epilepsy.

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Conflict of interests: Authors declare no conflict of interest.

Table 3. Effects of methanol extract of *B. orellana* on isoniazid-induced seizure in mice

Treatment	Quantal protection against seizure	% Protection against seizure	Mean onset of seizure ± SD (mins)	Quantal protection against mortality	% Protection against mortality
Polysorbate 80 (tween80)	0/5	0	3.35±1.00	0/5	0
Extract, 200 mg/kg	0/5	0	19.00±1.22****	0/5	0
Extract, 400 mg/kg	0/5	0	20.25±4.27****	0/5	0
Extract, 800 mg/kg	0/5	0	20.00±2.54****	0/5	0
Valproic acid 200 mg/kg	0/5	0	22.95±3.31****	2/5	33.3

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