Evaluation of anticonvulsant and antiepileptogenic activity of *Euphorbia nivulia* in PTZ-induced kindling model of epilepsy in mice

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Abstract: Epilepsy remains a major chronic neurological disorder with significantly higher refractory seizure rate. Based on the folk medicine literature, we explored the anticonvulsant and antiepileptogenic activity of aqueous ethanolic extracts of *Fumaria indica*, *Euphorbia lactea*, *Euphorbia helioscopia*, *Neurada procumbens*, and *Euphorbia nivulia*. The acute anticonvulsant activity of the extracts was determined at different concentrations in different groups of Swiss albino mice. Among all the materials tested, the ethanolic extracts of *Euphorbia nivulia* (eth-EN) alone was found to exhibit concentration-dependent anticonvulsant effects when evaluated against the acute convulsant dose of Pentylenetetrazole (PTZ, 90mg/kg, s.c.). eth-EN extract at 100mg/kg i.p concentration showed maximum protection against the PTZ induced mortality ($P<0.05$). eth-EN (100mg/kg) treated animals also showed significant reduction in the progression of epileptogenesis ($P<0.05$) when tested against the PTZ-induced (50mg/kg s.c.) chemical kindling model of epilepsy. The FT-IR spectra of this extract showed both known and unknown spectral peaks from which the presence of the functional groups; i.e. aromatics, diketones, alkenes, carbonyls, carboxylic acids and amide compounds were confirmed. The unknown peaks strongly suggested the presence of novel compounds that may be responsible for its anticonvulsant and antiepileptogenic activity.

Keywords: *Euphorbia nivulia Buch.-Ham.*, PTZ, epilepsy, seizures, FT-IR.

INTRODUCTION

Epilepsy is a major group of neurological disorders and widely spread worldwide. More than 2million Americans and 50million people worldwide are suffering from this disease (Strine *et al*., 2005) from which 80% population belongs to the low-income and developing countries (De Boer, 2002). The International League Against Epilepsy (ILAE) defines epilepsy as a transitory manifestation of signs or symptoms because of anomalous excessive or asynchronous neuronal bustle in the affected brain regions (Berg *et al*., 2010).

In Pakistan, 9.99 per 1000 people are suffering from epilepsy which is more common in rural areas as compare to the urban population (Khatri *et al*., 2004). About more than 0.7Million people with epilepsy are living in the Arab world (Benamer and Grosset, 2009). The Prevalence was found to be 0.90/1000 and 6.50/1000 in Sudan and Saudi Arabia, respectively with an average of 2.30/1000 (Benamer and Grosset, 2009). The prognosis of the newly diagnosed adult and adolescent epilepsies is considerably good after the introduction of various new antiepileptic drugs (AEDs) in the recent decades (Brodie, 2010, Kwan *et al*., 2010). However, the sustained seizure control still remains elusive along with a high rate of drug resistance in a large number of patient population (De Liso *et al*., 2016). The use of traditional and herbal medicine to cure the refractory conditions is gaining popularity and these phytomedicines are now used by more than 20% of world population (Bent, 2008). The use of medicinal plants fits into our culture because of the easy accessibility and affordability and with the least associated side effects (Khatri *et al*., 2004). Currently, more than 200-250 plant species are already in use in the Eastern Arab traditional medicine system for the treatment of various diseases (Lev and Amar, 2000, Lev and Amar, 2002, Said *et al*., 2002). A study had reported in 1982 that Pakistan had a wide range of remedial plants used for different ailments (Haq, 2004). For instance, our research group has already discovered and reported a very potent, novel anticonvulsant agent originally derived from a plant source with a promising efficacy profile (Ashraf *et al*., 2013).

Fumaria indica Pugsley is conventionally used for the management of dermatological disorders, cardio-vascular diseases, circulatory complaints, headache and fever (Murad *et al*., 2011). *Euphorbia lactea Roxb* is a fancy plant that makes hedges, it is also applied in traditional medicine against inflammatory tumor and warts (Al-Zanbagi *et al*., 2000). *Euphorbia helioscopia* Linn is employed as cathartic, hydrogogue and juice is useful for eruptions. Seeds of this plant are beneficial for the cure of cholera (Rahman and Akter, 2013). *Neurada procumbens* Linn is reported as a robust stimulating for weakness and

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impotency (Hameed *et al*., 2011). *Euphorbia nivulia Buch.-Ham* is expedient for the cure of dropsy, hepatomegaly and spleen and appreciated for hemorrhoids. Coagulated latex is utilized for bronchitis (Khare, 2003).

The aim of the current study is to analyze the anticonvulsant and anti-epileptogenic activity of the plants named: *Fumaria indica Pugsley, Euphorbia lactea Roxb, Euphorbia helioscopia* Linn., *Neurada procumbens* Linn. and *Euphorbia nivulia Buch.-Ham.* The selection of these plants was inspired by their use in the folk medicine practice to treat various disorders.

MATERIALS AND METHODS

Collection of plant materials and preparation of crude extracts

The plants i.e. *Fumaria indica Pugsley* (Herbarium No. FI-WP-04-14-85), *Euphorbia lactea* Roxb (EL-WP-04-14-83), *Euphorbia helioscopia* Linn. (EH-WP-04-14-84), *Neurada procumbens* Linn. (NP-WP-04-14-86) and *Euphorbia nivulia Buch.-Ham* (EU-WP-04-14-82) were collected from the rural areas of district Bahawalpur. The plants were identified by the expert botanist, Department of Botany, The Islamia University of Bahawalpur. Each plant was assigned with above mentioned herbarium number and the respective samples were stored for the future reference purposes.

The crude leaves of *Fumaria indica Pugsley* (Cr-FI), *Euphorbia lactea* Roxb (Cr-EL), *Euphorbia helioscopia* Linn (Cr-EH), *Neurada procumbens* Linn. (Cr-NP), and *Euphorbia nivulia Buch.-Ham* (Cr-EN) were cut down into small pieces after removal of the adulterated materials and draped for shade drying. On complete drying; each plant was subjected for grinding in an iron heavy grinder. For getting coarse powder, they were subjected to electric grinder. Then, the coarse powders were macerated in 70% ethanolic solution at room temperature (23-25^{o}C) for the period of fifteen days. The macerated materials were shaken thrice a day. The muslin cloth was used for the filtration of soaked material and then further filtration was performed by Grad-I Wattman filter paper. The filtrates were then vaporized in rotary evaporator under the influence of negative pressure (-760mmHg). A glutinous and semisolid pasty mass of each sample was achieved which was then placed in oven to acquire the utmost purity of the extract. The percentage yield was later calculated. Normal Saline (0.9%) and distilled water were used for the dissolution of crude extract for further experimental procedures (Gilani *et al*., 2005, Tona *et al*., 1998).

Pentylenetetrazole (PTZ; Sigma) and diazepam (a generous gift from Roche Pakistan Ltd.) were used for the convulsant test as well as in the chemical kindling model. The ethanolic extracts of *Fumaria indica Pugsley* (eth-FI), *Euphorbia lactea Roxb* (eth-EL), *Euphorbia helioscopia* Linn (eth-EH), *Neurada procumbens Linn* (eth-NP) and *Euphorbia nivulia Buch.-Ham* (eth-EN) were collected and dissolved in normal saline (0.9% NaCl) in a test tube and was shaken with the help of a vortex mixture which was then placed in ultrasonic shaker. Finally, the solution was filtered by using Grade-1Wattman filter paper.

PTZ-Induced seizure test

Adult, male Swiss-Albino mice weighing 20-30g were housed in a temperature & humidity controlled animal facility with a proper light/dark cycle. All the protocols and methods of the study were approved by the ethical committee of The Islamia University of Bahawalpur. The instructions of National Institute of Health Guide for the Care and Use of Laboratory Animals were followed for the conduction of all the experiments involving animals (Institute of Laboratory Animal Resources, 1996).

Anticonvulsant effects of plant extracts were observed *in vivo* by acute PTZ test as described by Simjee and coworkers (Simjee *et al*., 2012).The animals were divided into different treatment groups (n=6). The test materials of plant extracts, normal saline and diazepam were administered 30min prior to PTZ administration to their respective groups. After the administration of PTZ, the mice were obscured and observed for a period of 60min for the assessment of the absence or presence of different types of seizure patterns. The protection against the animal mortality testing material was also assessed against PTZ-induced mortality within a period of 24hours.

Diazepam, the drug of choice in status epilepticus, was used as a reference drug for the negative controls at the dose of 7.5mg/kg, i.p. (De Sarro G *et al*., 2000, Simjee *et al*., 2012).

Development of in vivo epileptogenic model

After the screening of the extracts in the acute seizure model, the active anticonvulsant test extract(s) was evaluated against the development of epilepsy process (epileptogenesis) using the PTZ chemical kindling model in mice. The well-established De Sarro method was employed for the induction of the kindling process (De Sarro G *et al*., 2000). For this purpose, the animals weighing 20-30g were divided into four groups, each consisting of eight animals/group (n=8). A sub-convulsive dose of PTZ (s.c. 50mg/kg) was administered every alternative day in all the groups except the normal controls. Moreover, 0.5ml normal saline (0.9% NaCl) and diazepam (7.5mg/kg, i.p.) were administered daily in the normal controls and negative controls, respectively. Similarly, the test extract of eth-EN (the one found active in the acute anticonvulsive test) was administered once daily in the test group. The test extract, normal saline and

Table 1: Duration of different seizures induced in different treatment groups. The plant extracts and Diazepam were injected i.p. 30min prior administration of PTZ (90mg/kg, i.p.). The values were represented as Mean ± SEM for the duration of different seizure patterns observed in test animals (n=6).

Groups	Treatment (mg/kg)	Onset of Jerks (sec)	Rear & Falling (sec)	HLTE (sec)	% Mortality
PTZ	90	99.6 ±3.16	132.3 ±3.91	247.8 ±5.12	100
Euphorbia nivulia	30	173.8 ±7.23	203.8 ±2.50	304.0 ±2.82	83.3
Euphorbia nivulia	50	265.6 ±13.33	334.1 ±3.77	650.5 ±24.75	66.6
Euphorbia nivulia	100	544.0 ±18.03	0	0	16.6
Fumaria indica	200	62.0 ± 2.08	125.3 ± 3.88	221.3 ± 1.76	100
Fumaria indica	400	72.17 ± 1.58	129.4 ± 3.7	229.2 ± 1.88	100
Euphorbia lactia	50	50.67 ± 1.23	113.3 ± 5.01	218.5 ± 4.77	100
Euphorbia lactia	100	57.33 ± 3.16	115.8 ± 4.85	223.4 ± 2.03	100
Euphorbia helioscopia	50	109.0 ± 1.91	162.5 ± 6.09	187.5 ± 4.64	100
Euphorbia helioscopia	100	130.33 ± 1.49	173.4 ± 5.32	198.3 ± 3.26	100
Neurada procumbens	250	61.0 ± 1.06	118.7 ± 2.73	178.6 ± 4.74	100
Neurada procumbens	500	66.67 ± 1.43	125.2 ± 5.92	183.3 ± 3.63	100
Diazepam	7.5	563.1 ±21.38	0	0	0
Normal Saline	0.25ml	-	-	-	-

diazepam were administered in their respective groups 30 min prior to the PTZ injection on the day of PTZ administration. The animals were placed for in observation chambers after each PTZ injection and observed for their behavioral seizure activity at least for 1hour. The seizure activity score was recorded for each animal according to the distinct pattern of seizure development scale as described elsewhere (Ashraf *et al*., 2013).

FT-IR Spectroscopic analyses

The Cr.EN was kept under the shade drying to remove moisture. The sample was ground in the mortar and pestle in order to obtain fine powder. The cellular constituents in this plant were monitored for the qualities of medicinal applications. Various functional groups present in the medicinal plants were identified. FT-IR (Tensor 27, Bruker, Germany) was used to record FT-IR spectra. The standard sample cell in FT-IR was a Pike Miracle single-bounce attenuated total reflectance (ATR) cell equipped with a ZnSe single crystal. A minute quantity of sample was directly placed on the small crystal spot and arm rotated over and turned down to press the sample down onto the crystal face for better contact and computerized for analyses by using the OPUS software (version6.5). The FT-IR analyses were performed for Cr.EN and eth.EN, respectively. The samples were scanned at room temperature at 4000 to 650cm^{-1} spectral range. Sixteen spectra per minute were recorded.

STATISTICAL ANALYSIS

Data collected after completion of experiments were evaluated and interpreted by SPSS as mean ± S.E.M. with 95% confidence interval (CI). Analysis of variance (ANOVA) with *post hoc* Tukey's test was employed for the data analysis. *P* values < 0.05 were considered significant. The resulting data were compared with the respective control groups and statistical analyses were performed by using Graph Pad Prism & SPSS.

RESULTS

Effects against PTZ-induced acute seizures in mice

Animals receiving concentrations (in mg/kg) 200 and 400 of eth-FI, 50 and 100 of eth-EL, 50 and 100 of eth-EH, 250 and 500 of eth-NP exhibited 100% mortality. However, it was observed that 30 and 50mg/kg of eth-EN showed 83.33% and 66.67% mortality, respectively, whereas it exhibited 16.67% mortality at the dose of 100 mg/kg (fig. 1).

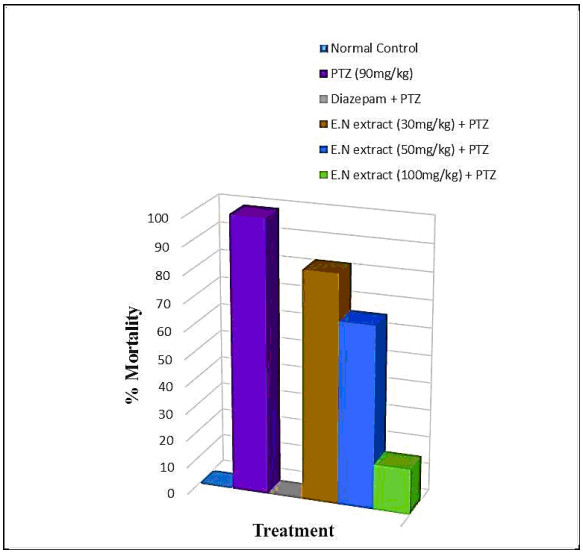

Fig. 1: Graphical representation of percentage mortality of *Euphorbia nivulia*. It was observed that 16.67%

mortality rate was observed at dose of 100mg/kg in eth-EN treated group. Whereas 66.67% and 83.33% mortality rate were observed at the dose of 50 and 30mg/kg respectively.

The onset of jerks was rapid in PTZ control group (99.6 ± 3.16 s), eth-FI treated group (62.0±2.08; 72.17±1.58 s), eth-EL treated group (50.67±1.23; 57.33±3.16 s), eth-EH treated group (109.0±1.91; 130.33±1.49 s), eth-NP treated group (61.0±1.06; 66.67±1.43s) and in normal saline group. eth-EN significantly delayed the onset at the concentration of 100mg/kg (544.0±18.03) as compared to the positive controls (P<0.05) which was comparable to that of diazepam (563.1±21.38 S) used as a standard drug as shown in table 1.

Similarly, the duration of rear and falling was rapid in PTZ control group (132.3±3.91 s), eth-FI treated group (125.3±3.88; 129.4±3.7 s), eth-EL treated group (113.3± 5.01; 115.8±4.85 s), eth-EH treated group (162.5±6.09; 173.4±5.32 s), eth-NP treated group (118.7±2.73; 125.2 ±5.92 s) and in normal saline group. Whereas eth-EN completely protected the onset of rear and falling at the concentration of 100mg/kg (P<0.05) also comparable to that of diazepam (table 1).

The duration of hind limb tonic extension (HLTE) was also achieved rapidly in PTZ control group (247.8±5.12 s), eth-FI treated group (221.3±1.76; 229.2±1.88 s), eth-EL treated group (218.5±4.77; 223.4±2.03 s), eth-EH treated group (187.5±4.64; 198.3±3.26 s), eth-NP treated group (178.6±4.74; 183.3±3.63 s) and in normal saline group. Whereas eth-EN completely protected the onset of HLTE at the concentration of 100mg/kg (P<0.05) similarly demonstrated by diazepam as shown in table 1.

These results show that, whereas the rest of the test extracts failed to protect the animals from mortality at all the tested concentrations, eth-EN fully protected stage 3 and onward convulsion patterns at 100mg/kg concen tration, and hence, showed significant anticonvulsant property with no apparent sign of toxicity to the animals.

Development of in vivo epileptogenic model

Based on the acute anticonvulsant activity, eth-EN was evaluated against the chemical kindling model of epilepsy in order to evaluate its possible effects to prevent the spread of seizures (anti-epileptogenic activity). The chemical kindling scores were measured as mean ± S.E.M of all the animals within a group (n=8). The treatment of 100mg/kg of eth-EN (the active test concentration as demonstrated in the acute PTZ model) showed significant anti-epileptogenic activity (seizure score of 2.0; P<0.05) as compared to the positive control PTZ-kindled group at the end of the kindling experiment as shown in fig. 2. The mice were considered fully kindled when the PTZ administered positive control group animals exhibited score 5, and the kindling experiment were stopped (fig. 2). It was observed that the animals in the positive controls (PTZ group) showed significant higher seizure activity than the test group after the fifth injection of PTZ onwards (P<0.001). Thus, the eth-EN administered group showed significant reduction in the development of seizure activity during the kindling experiment.

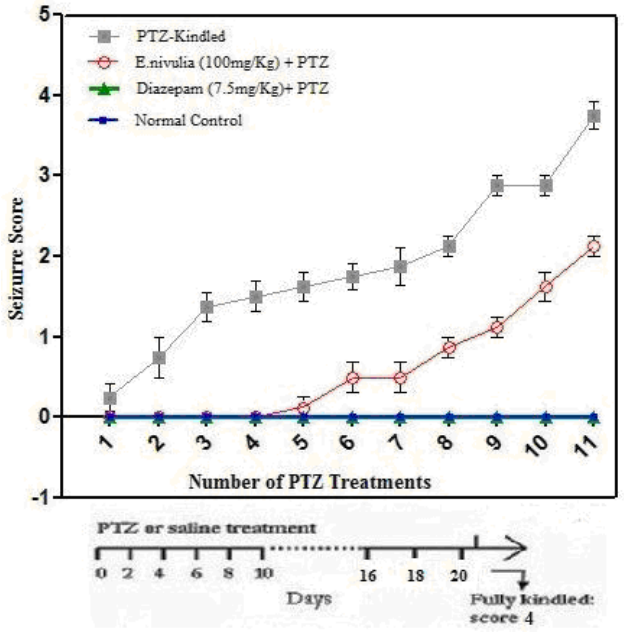

Fig. 2: Graphical representation of development of *in vivo* epileptogenic model. The values were expressed as mean± SEM of 8 mice of each group. The kindling scores were stated as the arithmetic mean±S.E.M. of 08 animals in each treatment group. The treatment of 100mg/kg of eth-EN (the active test dose as depicted in the acute PTZ model) showed significant anti-epileptogenic activity (seizure score of 2.0; P value=0.0001; F value=22.65) until the end of the experiment as shown in fig. 5. The kindling experiment was continued until the positive control group reached the score 5 when the animals were considered to be fully kindled (fig. 5). Starting from the fifth injection of PTZ, the average scores of PTZ-induced seizures were significantly higher in the kindled control rats (receiving no other treatment except PTZ).

FT-IR analyses of euphorbia nivulia buch.-ham

The spectroscopic analysis of cr-EN plant FT-IR analysis confirmed the presence of aromatics, amide, diketones, alkenes, carbonyls, carboxylic acids and amide compounds which shows major peaks for crude dried powder (fig. 3), while major peaks for dried ethanolic extract are indicated in fig. 4. The results of dried eth-EN plant FT-IR analysis revealed the presence of alkenes, aliphatic amines, alcohols, carboxylic acids, esters, aromatics, esters and alkenes. However, the compounds at 3734.86 and 3853.15 are still needed to be identified.

DISCUSSION

Current study was carried out for the scientific evaluation of *Fumaria indica Pugsley*, *Euphorbia lactea* Roxb, *Euphorbia helioscopia* Linn., *Neurada procumbens* Linn. and *Euphorbia nivulia Buch.-Ham.* which have great medicinal values in the folk medical literature. The focus of our study was to assess the anticonvulsant as well as anti-epileptogenic activity of these selected plants. Despite of considerable advances in the development of new AEDs especially during the last two decades, the permanent control of epileptic seizures remains a major medical challenge. It has been observed that the incidence of refractory seizures worldwide remains significantly higher (De Boer *et al*., 2008). For this purpose, we started to investigate these plant materials in order to develop the novel candidates for the effective control of epileptic seizures by using standard epilepsy model.

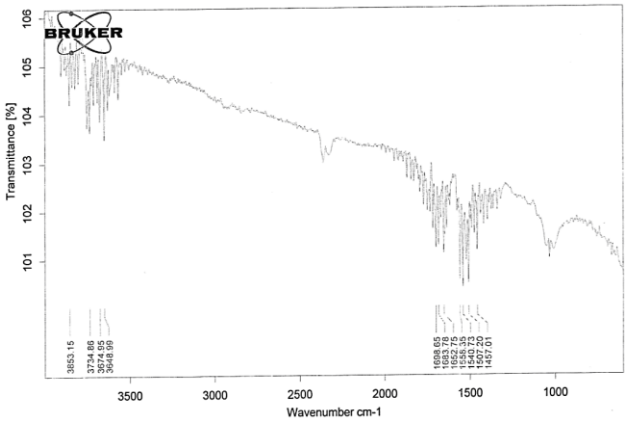

Fig. 3: FT-IR spectrum of crude dried powder of cr-EN.

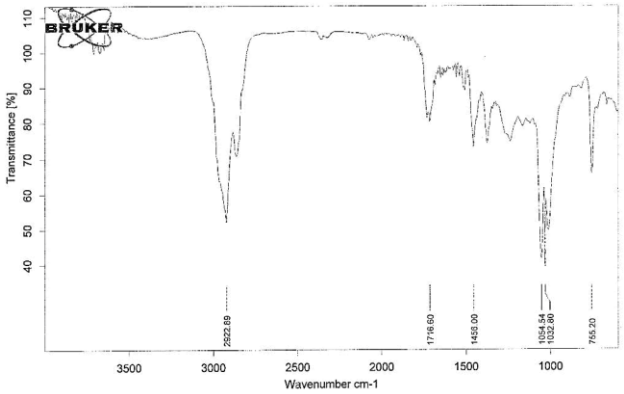

Fig. 4: FT-IR spectrum of eth-EN

From all plant extracts, only eth-EN exhibited significant anticonvulsant activity. *Euphorbia nivulia Buch.-Ham.* has the geographical diversity and distributed in the tropical regions of Asia, Europe, Africa and Australia. *Euphorbia nivulia Buch.-Ham.* is planted as hedge plant in the northern and central India, also often planted in dry areas and wild in the region of arid soils. eth-EN was injected (i.p.) and exhibited anticonvulsant effects against PTZ-induced convulsions in dose dependent manner as observed at doses 30, 50 and 100mg/kg respectively in different groups of Swiss albino mice. The exact mechanism of seizure induction by PTZ is unknown, however, it most likely inhibits the gamma aminobutyric acid (GABA; the major inhibitory neurotransmitter in the CNS) neurotransmission to produces seizures. The inhibition of GABAergic neurotransmission, among other factors, plays a vital role in the neuronal hyper excitability and hence disturbs the neuronal synchronicity that may lead to the development of epilepsy (De Sarro G *et al*., 2000, Amabeoku *et al*., 1998). It has already been widely reported that the increase in GABAergic neuro-transmission antagonizes seizures and vice versa (Leonard, 2003).

Diazepam (7.5mg/kg) was used as a reference drug in our studies. Since, diazepam exerts its anticonvulsant and antiepileptic effects by increasing the GABA neuro-transmission (Leonard, 2003), it was expected that it would protect the animals against PTZ-induced seizures in the negative controls, which it did.

While all remaining plant extracts were ruled out in the light of the acute anticonvulsant testing, we proceeded with chronic chemical Kindling model to evaluate the eth-EN against the development of epileptogenesis. For this purpose, the concentration of 100mg/kg of the plant extract was selected. At the end of experiment, the average score of 2.0 was recorded in eth-EN administered mice group as compared to score 5 in the PTZ kindled control group which means that it significantly slowed down the progression of epileptogenesis. Once the anticonvulsant and anti-epileptogenic activities of eth-EN were confirmed, next we analyzed the possible active chemical groups present in this medicinal plant.

FT-IR spectrum reflecting objectively the panorama of chemical components of plant extracts is a most credible method to validate and identify the mix-substance systems such as traditional medicine and herbal medicine (Baseri and Baker, 2011). The results of the present study spectrum also revealed the functional groups present in cr-EN and eth-EN. Many workers have applied the FTIR spectrum as a tool for discriminating, classifying and differentiating closely related plants. Therefore, the present work on *Euphorbia nivulia Buch.-Ham.* showed different phytochemical markers as useful analytical tool to check not only the quality of the powder and extracts but also to identify the medicinally important plant. Further advanced spectroscopic studies are needed for the structural elucidation and identification of various beneficial compounds present. The cr-EN and eth-EN were subjected to FT-IR analysis for the detection of different functional groups present in *Euphorbia nivulia Buch.-Ham.* The FT-IR study showed the similarities as well as the variations among the cr-EN and

eth-EN based on the transmittance spectrum and presence of various functional groups. Because of the similarity of the extraction method by Rotary Evaporator, the chemical components in the concentrated extracts are relatively consistent and therefore they show higher comparability and repeatability in the FT-IR spectra. Results of FT-IR spectroscopic analysis in the cr.EN and eth-EN have revealed the presence of various chemical constituents. This study revealed some functional groups with different spectral peaks from which we can assess the chemical and structural nature of compound(s) having anticonvulsant and antiepileptic activity. The values at 1457.01, 1507.20, (1540.73, 1558.35), 1652.75, 1683.78, 1698.65, (3648.99, 3674.95), 3734.86 and 3853.15 attributed aromatics, amide, diketones, alkenes, carbonyls, carboxylic acids and amide compounds, respectively in the crude powder. While the dried eth-EN plant FT-IR analysis revealed the presence of alkenes, aliphatic amines, alcohols, carboxylic acids, esters, aromatics, esters and alkenes which shows major peaks at 755.20, 1032.80, 1054.54, 1456.00, 1716.60, and 2922.89, respectively.

The current study showed that the eth-EN exhibited significant anticonvulsant and antiepileptogenic activity which could be due to the presence of compounds. However, the plant extract should be subjected to fractionation and isolation of the active constituent(s), elucidation of their chemical structure and exploration of possible mechanism(s) of action. This inceptive study may tile the way for further scientific authentication of this medicinal plant for developing novel antiepileptic drug(s) with better efficacy.

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