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Evaluation of gastrointestinal motility, anticonvulsant activity, and antioxidant activity of a Bangladeshi medicinal plant, Diospyros peregrine, in mice

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ABSTRACT:
The study of free radicals, gastrointestinal motility, and anticonvulsants in biology is producing a medical revolution that pledges a new era of health and disease management. The current study aimed to investigate the antioxidant activity, gastrointestinal motility activity, and anticonvulsant activity of Diospyros peregrina root extract at concentrations of 200 and 400 mg/kg .DPPH and total phenolic compounds were used for the analysis of antioxidant activity, where Gallic acid was used as a standard for the determination of total phenolic content by the modified Folin-Ciocaltu method. The crude methanol extract exhibited a significant amount of total phenolic compound in the root (639.25 in roots).Further, the gastrointestinal motility test showed the significant activity of the methanolic root extract of Diospyros peregrina. The presence of charcoal inside the intestine after 45 minutes of feeding proves that the extracts of the root of Diospyros peregrina atboth concentrations of 200 mg/kg and 400 mg/kg body weight in mice showed the percent of gastrointestinal motility to be 58.43 and 71.54. Furthermore, the methanolic crude root extract was screened for anticonvulsant activity using mice. The methanolic extract possesses fictive scratching, head noodling, and tremor. The methanolic extract showed a 100% protection rate.
Keywords: Diospyros peregrina, antioxidant, Folin-Ciocalteau, DPPH, gastro-intestinal motility, anticonvulsant.

I. INTRODUCTION:

Middle-sized D. peregrina (Ebenaceae) is a small tree that grows luxuriantly in the plains of West Bengal on the coastal side. The whole plant is used in fever, snake bite, wound and ulcer healing. It is also reported to possess antifertility, hepatoprotective, hypoglycemic, antiviral, and antiprotozoal activities (1).The fruit of this plant can be used for medicinal purposes as ripe fruits are edible with ethnomedicinal significance as tonic and aphrodisiac(2). Unripe fruits are used to treat diarrhoea, dysentery, cholera, ulcer of the mouth and wounds(3). Fruits contain various phytochemicals like triterpenes, alkanes, flavonoids and tannins (3,4). The dried unripe fruits of D. peregrina are also indicated to control diabetes in coastal west Bengal by villagers. Different parts of the plants are also used traditionally for the treatment of dysentery and menstrual problems (3,5).

For many years much endeavour has been dedicated to natural antioxidant and their affinity with health advantages (6). Plants are dynamic sources of natural antioxidants that can counteract various reactive oxygen species (7). Various forms of ROS are found, which include free radicals' species eg. Superoxide anion radicals (O2 -), hydroxyl radicals (OH.) and non-free-radical species eg. H_2O_2 and singled oxygen (1O2). Such elements are exacerbating factors in cellular damage and the aging process (8).In foods, ROS cause deterioration by promoting lipid peroxidation reactions(9). Phenol-containing plants are commonly found in both edible and non-edible plants and are reported to have multiple biological effects like antioxidant activity. Due to their redox properties, phenols function as reducing agents and oxygen quenchers. Moreover, they have the efficacy of showing a metal chelation potential (10). Various studies have been done to assess the correlation between phenolic compounds and antioxidant activity (11).

Epilepsy is known as a major neurological disorder, and up to 5% of the world's population has epilepsy in their long life. The current epilepsy treatment with modern antiepileptic drugs reveals side effects, dose-related and chronic toxicity, and also causes teratogenic effects; With the current antiepileptic treatment system, approximately 30% of the patients' experience seizures (12). A large number of populations (80) in growing countries

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rely on traditional medicines or folk remedies (13). An unequal distribution of the excitatory and inhibitory neurotransmitters is thought to be responsible for seizures. Glutamatergic excitatory neurotransmitters influence seizure activity at the neuronal level when it overrides gamma-aminobutyric acid (GABA) mediated inhibition (14). Many drugs which can enrich the brain contents of GABA have to exert anticonvulsant activity against seizures which can be induced by isoniazid and Pilocarpine (Pilo). Secondary metabolite of several botanic is used to treat psychotic disorders, especially for traditional medicinal practice, which can affect the central nervous system, noradrenaline, serotonin, GABA and BDZ neurotransmitters activity directly or indirectly (15).

Gastrointestinal (GI) disorders are a common reason for medical care and have a significant impact on quality of life. Diarrhea is one of the gastrointestinal disorders that cause infant deaths, especially in poor countries (16). However, about 20% of total children die from diarrhea before they reach the age of five in developing countries (17). Various synthetic anti-diarrheal drugs are available in the market, although most of them have side effects like uncomfortable bowel movements, uneasiness, etc. Hence there has been a regular exploration of drugs that might prohibit diarrhoea without unwanted adverse side effects and as the object of oral dehydration therapy(18).

II. MATERIALS AND METHOD:

Drugs, Chemicals and Reagents

Methanol was purchased from Merck, Darm-stadt, Germany; Castor oil was purchased from WELL's Heath Care, Spain. Diazepam(Square Pharmaceuticals Ltd; Dhaka, Bangladesh). Pilocarpine hydrochloride **(**Popular Pharmaceuticals Ltd. Bangladesh), Isoniazid(Novartis (Bangladesh) Ltd.), Hyoscine butyl bromide(ACI Ltd. Bangladesh), Activated Charcoal (Lab. Reagent, India), DPPH (1,1-diphenyl, 2-picryl hydrazyl), trichloroacetic acid, ferric chloride, Gallic acid and BHA were obtained from Sigma Chemical Co. USA. Folin-Ciocalteu reagent, sodium carbonate and potassium ferricyanide were purchased from Merck, Germany. Hydrochloric Acid (BDH Ltd, England), Chloroform (ACS, Merck), Ammonia (Merck Millipore, India), Ferric Chloride (Fisher, USA). Acetic anhydride, Sulphuric acid, lead acetate, Nitric acid, and Copper acetate were also purchased from Merck, Darmstadt, Germany. All other reagents were procured from Sigma Chemicals limited.All of the chemicals utilized were of analytical reagent quality.

Laboratory Animals

Swiss-albino mice of either sex, aged 4-5 weeks, weighing -18-25gm, obtained from the Laboratory mice-breeding centre, the department of pharmacy, Jahangirnagar University, were used for the studies. The animals were kept in well-ventilated and hygienic compartments maintained under standard laboratory conditions (relative humidity 55-65%, room temperature 23.0±2.0° C and 12 h light: dark cycle).The animals were fed with a standard controlled diet, and access to water ad libitum was timely.All animal studies were conducted in accordance with the regulations of the NSTU Research Cell Animal Ethics Committee, Noakhali Science and Technology University. These guidelines were in accordance with the internationally accepted principles for laboratory use and care.

Plant material:

The roots of D. peregrinawere collected from Daudkandi, Comilla, Bangladesh. A voucher specimen for this plant has been collected from Bangladesh National Herbarium, Dhaka, Bangladesh (Accession no. 39516). The dried roots were ground into coarse powder by a hammer mill. The dried and powdered plant material was subjected to maceration by methanol (MeOH) at room temperature. Using a rotary evaporator, the solvent was evaporated from the filtered solution.

Preparation and extraction:

Cold maceration technique was used for extraction. The dried and powdered roots (500g) were soaked in 1800 ml of methanol for about 15 days at room temperature with regular stirring. The solution was filtered after 15 days by using filter cloth and Whatman® filter paper No. 1 (Sargent-Welch, USA) and then evaporated using a rotary evaporator until dried. When it became rendered into a brown granular compound, the brown granular compound was designated as a crude extract of methanol.

Antioxidant activity:

DPPH free radical scavenging activity:

The method established by Braca et al. 2001 was followed to determine the free radical scavenging activity of the extract based on the scavenging activity of the stable 1, 1- diphenyl-2-

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picrylhydrazyl (DPPH) free radical. A small amount of methanol (2.0 ml) solution of the extract was mixed with different concentrations (500 to 0.977 µg ml) and 3.0 ml of a DPPH methanol solution (20µg/ml). After 30 minutes of the reaction period, the absorbance was calculated at 517 nm against methanol as blank by UV spectrophotometer at room temperature. The percentage inhibition activity was gained by using this equation, [(A0–A1)/A0] ×100, where A0 express the absorbance of the control, and A1 indicates the absorbance of the extract/ standard. Then IC50 values were calculated after the inhibition curves were ripened. BHT was used as a positive control.

$$\% \textbf{ radical scavenging activity} = \frac{\textbf{A control} - \textbf{A test sample}}{\textbf{A control}} \times \textbf{100}$$

Here, A stands for absorbance.
The graph plotted with inhibition percentage against extract/standard concentration; extract concentration providing 50% inhibition (IC_{50}) was calculated.

Total phenol and flavonoid content:

The total phenolic content of the extract was evaluated according to the method descried by Harbertson and Spaydet al.(19). Folin-Ciocalteu method was used to determine the total phenolic content of the root extract. This method was used to mix extract (0.5 mL) with 0.5 mL of 1:1 diluted Folin-Ciocalteu reagent along with 4 mL of sodium carbonate (1 M). The mixtures were kept standing for 15 minutes, and the total phenol content was determined calorimetrically at 760 nm. A standard curve was prepared by using an increasing concentration of gallic acid in methanol. Different concentrations of gallic acid (standard, 0-100) µg/mL) was used to plot a standard curve. Total phenolic content was estimated as µggallic acid equivalents (GAE)/mg of extract. Total flavonoid content was determined following a method by Saeed et al. (20). The absorbance was measured at 506 nm using UV spectroscopy.

Gastrointestinal (GI) motility test:

The method described by Abdullahi et al., 2001. was resorted to studying the effect of D. peregrina root extract on gastrointestinal transit in mice. The test animals were famished for 24 h prior to the experiment, but access to water was maintained. Two divided groups (control and test group) contained five mice in each group. The vehicle (1% Tween 80 in water) was administered orally at a dose of 0.01ml/mg body weight to the control group. Test groups received the root extract of D. peregrina at doses of 200 and 400 mg/kg body weight. After 30 min, mice of each group were fed 1ml of the charcoal meal (3% charcoal suspension in 5% gum acacia). After 30 min of the administration of charcoal meal, the animals of each group needed to be sacrificed, and the length of the intestine (pyloric sphincter to caecum) as well as the distance traversed by charcoal as a fraction of that length was measured. The charcoal movement in the intestine was expressed as a percentage.

The percent motility was assessed by using the following formula:

$$\% \text{ of of Motility Inhibition} = \frac{T0 - T1}{T0} \times 100$$

Where T0= Total length of intestine
T1= distance covered by charcoal

Anticonvulsive test:
Epileptic behaviours:

Behavioural seizures were classified as follows: class A, hypoactivity and mouth and facial automatisms; class B, head nodding and mastication; class C, forelimb clonuses without rearing; class IV, bilateral forelimb clonuses and rearing; class D, rearing and loss of posture. Mice were observed continuously for 30 minutes following pilocarpine administration for the occurrence of limbic seizures and status epilepticus. The latency to the first episode of convulsive behaviour (forelimb clonus) and the latency to the onset of status epilepticus was also recorded.

Isoniazid (INH) Induced Convulsions:

Albino mice (18-22 g) of either sex were randomly divided into four groups of five mice each (n=5) and were fasted overnight prior to the test, but the water was supplied at libitum. Group I was maintained as control and was given INH (300 mg/kg i.p.) only, Group II- Standard received Diazepam (5mg/kg i.p.), Group III- tested with methanolic roots extract 200mg/kg, and Group IV- receives methanolic roots extract 400mg/kg. The extract was administered i.p. to groups of mice at doses of 200 and 400 mg/kg.i.p 1h before administration of INH (300 mg/kg, i.p.) whereas, in

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Group II INH was injected after 30 min of diazepam (5 mg/kg, i.p) treatment. The mice were placed in an isolated perplex chamber and observed for the following 30 mins for the onset of clonic seizures, an extension of the hind limb, Fictive scratching, Tremors, Stupor and percent protection. The percentages of seizures or deaths occurring in the control group were taken as 100%. The concealment of these effects in the treated groups was assessed as a percentage of controls.

Pilocarpine Induced Convulsions:

Pilocarpine hydrochloride was dissolved in distilled water and administered i.p. to induce seizures. After 1 hour of injecting extracts, Hyoscinebutylbromide (1 mg/kg in distill, i.p.) was administered (5 ml/kg i.p). Peripheral effects were minimized by the administration of Pilocarpine after 30 minutes of Hyoscine butyl bromide. The latency to the first convulsion and the latency and percentage mortality was recorded for a period of 30 min. Animals surviving more than 30 min were considered to be protected. The latency to the first episode of convulsive behaviour (forelimb clonus), and the latency to the onset of status epilepticus were also recorded.

Statistical analysis:

Results were revealed as mean ± standard error of the mean(S.E.M.). Statistical analysis was performedby using one-way analysis of variance (ANOVA), followed by a student t-test.

III. RESULT:

Antioxidant Test Analysis:

DPPH Free Radical Scavenging Assay:

The root extracts produced significant DPPH radical scavenging activity from 10mg/ml (Figure 1). The DPPH antioxidant assay is based on the ability of DPPH. The presence of antioxidants causes decolorization of a stable free radical. The antioxidant activity of D. peregrina was compared with Butylated hydroxy anisole (BHA) (standard). In this investigation, the crude methanolic extract of D. peregrina roots showed free radical scavengingactivity with an IC_{50} value of 0.0016±.0014 mg/ml, and the maximum inhibition was observed as 88.31% (As shown in fig 1). Butylated hydroxy anisole (BHA) was also used as a standard compound to study the DPPH radical scavenging activity. The Figure-1 shows the DPPH scavenging activity of BHA. It showed maximum inhibition 93.09±0.019 at 100 µg/ml, and 50% inhibitory concentration (IC_{50}) was found at 4.10±0.03.

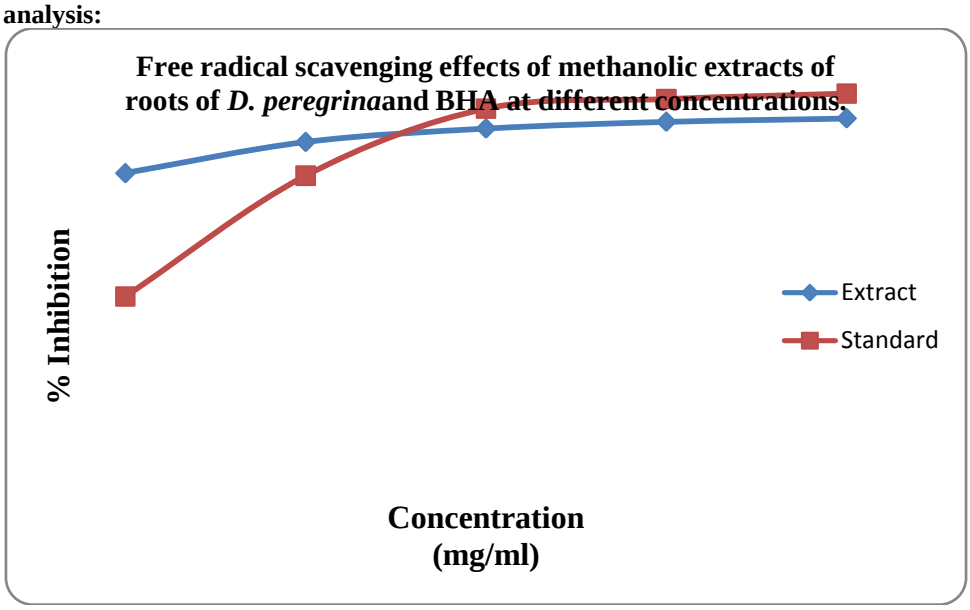

Figure 1:DPPH radical scavenging activity of venging different concentrations of methanol extracts of roots of D. peregrina and BHA at different concentrations.

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Total phenolic and flavonoid content analysis:

Phenols and flavonoids present in the plant root extract may be responsible for the antioxidant action in the tested models. The result (Table 1) is expressed as the number of gallic acid equivalents per gram of the plant extractives. The total phenolic content found to be a high amount in the roots extract of D. peregrinais 2.66±0.027mg gallic acid (GAE)/gm extract (Table 1). The result is expressed as the number of equivalents to Gallic acid per gram of the plant extract. The plant extract was found to contain a large amount of phenolic content. The plant extract demonstrated moderate total antioxidant capacity.

Table 1: Total phenolic content determination of methanolic extract of roots of Diospyros peregrina.

	Total phenolic content (mg GAE equivalent per gm of dry extract, mean±SEM)	Total flavonoid content (mg quercetin equivalent per mg, mean±SEM)
Methanol extract of Diospyros peregrina (roots)	2.66±0.027	1.29±0.063

Data represent mean ± standard error mean (n=3) of duplicate analysis

Gastrointestinal motility (GI) activity test:

The distance roofed by the charcoal plug is shown in Table 2. Comparisons indicate that loperamide significantly decreased the appearance of charcoal travelled in the feces when roots extract of D. peregrinais used. The drug used as a positive control was loperamide (Imosec™, Janssen-Cilag). The investigation was carried out to determine the effects of this positive control on Gastrointestinal (GI) motility. The result shows high evidence that this drug caused a significant decrease in gut motility compared with the effect produced by normal saline. The distance covered by charcoal in treated mice and the distance covered by charcoal in control mice were closely related. The marked decrease in the propulsive movements induced by loperamide could be determined by the observed appearance of charcoal in the animal feces. We can see that in control mice, the percent motility is 42.67%, whereas, in the treated mice, the percent inhibition is 58.50% at 200mg/kg concentration and 71.54% at 400mg/kg concentration (Table 2).

Table 2: Effects of D. peregrine root extracts ongastrointestinal motility in mice.

Treatment	**Dose**	**The total length of intestine Mean ±S.E.M**	**Distance covered by charcoal Mean ±S.E.M**	**% Of motility inhibition**
Control	10ml/kg	58.00±1.00	33.25±0.17	42.67
Castrol oil	10ml/kg	61.83±1.77	30.40±1.43	50.83
D. peregrine (root)	200 mg/kg	65.51±2.101	26.71±3.36	58.43
	400 mg/kg	66.12±1.93	18.79±1.26	71.54

Values are expressed as mean±SEM (n=5).

Anticonvulsant Activity Test:

Isoniazid-induced seizure:

A 300 mg/kg of subcutaneous isoniazid-induced tonic-clonic seizures in all tested animals (Table 3). The root extract group and the standard group were compared with the control group. A dose of 200 mg/kg p.o. of root extracts delayed the onset of convulsion (378.2±142.43) and reduced the duration of action of convulsion (36.6±13.37) induced by isoniazid. A dose of 400 mg/kg i.p. of D. peregrina, delayed the onset of convulsion (738.16±322.23s) and reduced the duration of convulsion (6.4±2.12 s) in mice against INH-induced convulsion. Both doses of extracts showed 100% protection against INH-induced convulsion in mice. On the other hand, in control, the percentage of mortality is 100 (5 died out of 5 mice. The typical anticonvulsant, diazepam 10 mg/kg i.p., eliminated the effects of isoniazid-induced convulsions in mice and provided complete protection.

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Table 3: Effect of methanolic extract of roots of D. peregrina in isoniazid-induced convulsion in mice.

Experimental group	Dose mg/kg b.w	Onset of clonic convulsion(s) Mean ±S.E.M	Duration of convulsion(s) Mean ±S.E.M	% of protection
Control (2% tween 80)	10 ml/kg (p.o)	254.06±102.50	9.6±2.58	0
Standard (diazepam)	5.0 mg/kg (i.p)	0	0	100
Diospyros peregrina(roots)	200 mg/kg (i.p)	378.2±142.43	36.6±13.37	100
	400 mg/kg (i.p)	738.16±322.233	6.4±2.12	100

All values are expressed as Mean ±S.E.M, n=5 mice in each group, tested by one-way ANOVA followed by Dunnett's Multiple Comparisons Test. (compared with the control group).

Pilocarpine-induced seizures:

In Pilocarpine (240mg/kg body weight) induced convulsion test, all the mice in the control group exhibited generalized limbic seizures after pilocarpine administration at a latency 4.57 seconds in root at 200mg/kg (Table-4). From Table4, we observed that, at a dose of 200 mg/kg of D. pereginaroot extract, it significantly reduced the onset of clonic convulsion (194.75±89.35) but couldn't reduce the duration of convulsion (4.57±2.03) with compared to control on mice. The methanolic extract at a dose of 400 mg/kg reduced the onset of clonic convulsion (303.50±112.64)and duration of convulsion(2.89±1.87) compared to the control(132.8 ±14.99, 6.2±2.77respectively). On the other hand, the methanolic extract reduced protected 100% at both doses, but in control, the percentage of mortality was 100 (5 died out of 5 mice). The onset of clonic convulsion andduration of convulsion was totally abolished in standard with compared to control and extract. There was no death of mice observed in the standard drug diazepam.

Table 4: Effects of methanolic extract of roots of Diospyros peregrina on Pilocarpine-induced convulsions in mice.

Experimental group	Dose Mg/kg b.w	Onset of clonic convulsion(s)Mean ±S.E.M	Duration of convulsion(s) Mean ±S.E.M	% of protection
Control (2% tween 80)	10 ml/kg (p.o)	132.8 ± 14.99	6.2± 2.77	0
Standard (diazepam)	5.0 mg/kg (i.p)	0	0	100
Diospyros peregrina(roots)	200 mg/kg (i.p)	194.75±89.35	4.57±2.03	100
	400 mg/kg (i.p)	303.50±112.64	2.89±1.87	100

All values are expressed as Mean ±S.E.M, n=5 mice in each group, by one-way ANOVA followed by Dunnett's Multiple Comparisons Test. (compared with the control group).

IV. DISCUSSION:

There is growing evidence that native antioxidants can protect against oxidative stress, and there is growing interested in the biochemical qualities of natural antioxidants found in herbs, spices, and medicinal plants (21).

There are various mechanisms by which antioxidants can inhibit the lipid peroxidation reaction. One of them is by scavenging reactive oxygen and nitrogen free radicals. Free radical scavenging activity can be enumerated by several types of practical methods. Among them, totally free radical scavenging can be evaluated by ascertaining their efficiency in scavenging DPPH radicals. This method depends on the reduction of DPPH, a stable free radical and any molecule which is able to donate an electron or hydrogen to DPPH can react with it and thereby whiten the DPPH absorption. DPPH shows a strong absorption maximum at 517 nm because of its odd electron by visible spectroscopy (purple colour). When a hydrogen donor, such as a free radical scavenging antioxidant, pairs off the free radical, the strength of absorption decreases, and decolourization is stoichiometric with regard to the number of electrons grabbed(22). The hydroxyls present in phenol are responsible for the radical scavenging

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effect, generally due to redox properties (10). According to our study, the high phenolic content present in D. pereginacan interpret as its high free radical scavenging property. This study discloses that the tested part of D. pereginahas moderate to significant antioxidant activity and free radical scavenging activity. According to the acquired results, we can suggest that the root of D. pereginaplants can be used as a source of antioxidants which is well demonstrated.

The results from this investigation pointed out that the methanolic extract of D. peregina possesses antidiarrhoeal characteristics. The root extract of this plant may have different agents that practically reduced diarrhea that was induced by a potent diarrhoeal agent, castor oil. Diarrhea can be identified by different phenomena, including frequent outflow of liquid (waterish) feces, high intestinal motility, high deposition of beneficent nutrients in the lumen of the intestine, and others (23). The findings from the test are in consent with previous works (24). As reported, the anti-diarrheal properties of plant extracts are disclosed by their reducing action of intestinal motility and augmenting intestinal reuptake, which is actually done by inhibiting the prostaglandin release (25–27). A high rate of intestinal absorption may lead to a decrease in intestinal deposition and, together with reduced intestinal motility, may result in increased transit time (28). Hence, the found anti-diarrhoeal activities of D. Peregrina in this study might be due to the grab of chemicals that simplify the aforesaid actions. Phytochemical groups like flavonoids, tannins, alkaloids and saponins have been reported to show ant diarrhoeal activities (29).

In isoniazid-induced convulsions, GABA content decreased below a critical level in some neurons and lower the GABA content in the brain to approximately the same extent in rats and mice (30). Standard drug diazepam showed a marked protective effect against isoniazid and Pilocarpine-induced convulsions in mice (31). In INH-induced convulsion, root extracts showed a protecting effect in mice by significantly delaying the onset of the seizure and reducing the duration of convulsion, which indicates the presence of anticonvulsant compounds that may produce an effect by enhancing GABAA receptor-mediated inhibition, as standard drugs like benzodiazepines enhance GABAA receptor-mediated inhibition in mice. During Pilocarpine-induced seizure, lipid peroxidation levels are increased in adult rats, which indicates the involvement of free radicals in the PILO-induced brain damage (32). Pilocarpine produces repetitive limbic seizures and status epilepticus in mice and replicates several features of human temporal lobe epilepsy (33). In the present work, the methanolic extract of D. peregrina roots produced 100% protection of mice against isoniazid and Pilocarpine-induced seizure. The protection of the extract indicates that the extract interacts with GABA-ergic neurons (34).

Phytochemical studies indicate the presence of alkaloids, steroids, flavonoids, saponins, proteins and tannins in D. peregina extracts. Previous reports provide that alkaloids, flavonoids, saponins, and triterpenoids are attributed to anticonvulsant activity in some experimental seizure models(35–37).Various reports showed that certain antioxidants have anticonvulsant activity against PILO-induced seizure. It is also found that flavonoids modulate GABA-generated chloride currents like benzodiazepine- molecules in animal models of anxiety and convulsion (38,39). Thus, the ability of D. peregina to attenuate seizures induced by PILO in rats could be attributed to the anticonvulsant effect by increasing GABA and/or its receptor densities or through antioxidant mechanisms (40).

V. CONCLUSION:

The activities of the extract may be multiplicated to the availability of various phytochemicals. The methanolic extract of D. peregrina roots produced 100% protection of mice against isoniazid and Pilocarpine-induced seizure. The protection of the extract indicates that the extract interacts with GABA-ergic neurotransmission. In conclusion, the findings of this study notify that the methanol extracts of D. peregrina roots contain a high degree of antioxidant properties and bioactive principles that may be of beneficent in the treatment of epilepsy and diarrhea.

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This work was a collaborative effort among all authors. Author MFM performed the statistical analysis and wrote the first draft of the manuscript. LB supervised and designed the whole manuscript, PC contributed in managed the literature searches and the linguistic improvement and revising of the whole manuscript.

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