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GCMS Analysis of Phytoconstituents in Leaf Extract of *Drypetes roxburghii* and its Neuroprotective Potential

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ABSTRACT: The study deals with the determination of neuroprotective effect of crude extract *Drypetes roxburghii* leaves. In this study, the crude extract of leaves of *D. roxburghii* was obtained and the phytochemical composition was analyzed using GC-MS. Afterwards, the acute oral toxicity of the extract was performed using OECD 420, based on which two doses, 100 and 200 mg/kg BW, were selected for further pharmacological evaluation. The Neuroprotective activity of the extract was evaluated in regard to its anti-epileptic, anti-anxiety, anti-depressant, muscle relaxant, and anti-oxidant activity. The mentioned activities were performed using various experimental animal models. The data was analyzed using graph pad prism, version 9. P<0.05 was selected as criteria of statistical significance. The results of the study revealed that the crude extract was found to be rich in many medicinally important constituents such as 5-ethylhydantoin, cis-vaccenic acid, and phenol, 2, 4-bis (1, 1- dimethylethyl). The extract possessed significant anti-epileptic, anti-anxiety, muscle relaxant, and anti-oxidant activity but not anti-depressant activity. The effect of test dose 200 mg/kg, BW was comparable to that of standard drug. Based on the result it can be concluded that the crude extract of *D. roxburghii* leaves contains constituents responsible for its neuroprotective potential.

Keywords: *Drypetes roxburghii*, GCMS analysis, anti-epileptic, anti-anxiety, anti-depressant, muscle relaxant, and anti-oxidant activity.

INTRODUCTION

The term "neuroprotection" refers to the methods and supporting mechanisms that can protect the central nervous system (CNS) from the neuronal damage brought on by a variety of neuropsychiatric and neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, anxiety, cerebrovascular impairment, seizures, and others. Neurodegenerative disorders are expected to be the second leading cause of death among people over 65 by the year 2040 (Kumar *et al.,* 2015). Complementary and alternative medicine (CAM) has grown in popularity in recent years. Many plant species have evolved as herbal remedies, and the active components of these plants have been the subject of intensive scientific investigation all around the globe. CAM or traditional therapies are thought to be safe and helpful in sensitive and complex conditions such as CNS disorders, with fewer adverse effects than synthetic medications. More than two-thirds of the active compounds lately released onto the market have some link to natural sources, whereas just 30% of the novel chemical entities utilized as medications are totally manufactured (Uddin and Zidorn 2020).

Herbal treatment is globally trusted for alleviating neurological ailments and disorders. A plethora of traditional medical systems, including Ayurveda, traditional Chinese medicine (TCM), Korean, Japanese, and others, have extensively recorded a multitude of medicinal plants that facilitate memory enhancement. According to Ayurveda, certain medicinal herbs such as Ashwagandha, Jatamansi, Vacha, Shankhapushpi, Kavach beej, Bramhi, and Mandukparni enhance intelligence by elevating the levels of neurotransmitters, notably acetylcholine, in the brain and enhancing the flow of blood in the cerebral region.

Plant derived chemicals impart neuroprotection by counteracting against oxidative stress, mitochondrial dysfunction, neurotrophic factor deficit, apoptosis and abnormal protein accumulation via regulation of mitochondrial apoptotic machinery, modification of cellular signal pathways, and induction of the anti-apoptotic Bcl-2 (B-cell lymphoma-2) protein family (Naoi *et al.,* 2019).

Drypetes roxburghii (Wall.) Hurus is widely distributed in the United States, India, Papua New Guinea, Taiwan, and Trinidad and Tobago. It has been said to have therapeutic properties, such as leaves being used to cure catarrh, skin illness, fever, cold, rheumatism, and infertility; the fruit, seeds, and leaves being used as an aphrodisiac, a tonic to help conception, and for the treatment of filarial disorders. Among the tribes of Uttar Pradesh, India, dispersed leaves are strewn on the maternity room floor to facilitate birth, and a garland of dried seeds is worn to protect against red pimples and allergies.

The *Drypetes* species, is found to be rich in sesquiterpenes, triterpenes, diterpenes, flavonoids, lignans, phenylpropanoids, steroids, and thiocyanates and is reported to have *in-vitro* and *in-vivo* analgesic, anthelmintic, antidiabetic, anti-emetic, anti-inflammatory, antioxidant, antiparasitic, central nervous system depressant, cytotoxic, and insecticidal activities (Wansi *et al.,* 2016).

To the best of our knowledge, the neuroprotective activity of leaf extract of *Drypetes roxburghii has* not been studied yet. Therefore, the study was aimed at the extraction of leaves of *D. Roxburghii,* finding the phytoconstituents present in the extract using GC-MS analysis, and evaluation of its neuroprotective potential.

MATERIALS AND METHODS

A. Plant material

Leaves of *Drypetes roxburghii* was collected locally and authenticated from Botany department of Bilwal Medcham and Research Laboratory Pvt. Ltd. Jaipur, Rajasthan, India.

B. Chemicals

Diazepam (Valium tablet, Abbott healthcare ltd., H.P, India), flouxetine (Flunil suspension, Intas Pharmaceutical ltd., Ahmedabad, Gujrat, India), Pentylenetetrazol, 5-Hydroxy tryptamine, beta-phenylalanine, Dichlorvos and Griess reagent were purchased from Sigma Aldrich (USA, MO); Carboxy methyl cellulose, **sucrose,** Tris-HCl, EDTA disodium salt dihydrate, hydrochloric acid, cyclohexane, butyl acetate, Quercitin dihydrate, sodium chloride, ethanol, petroleum ether, potassium dichromate and acetic acid, tricholoacetic acid, thiobarbituric acid, sulphosalicylic acid, DTNB (5,5, dithiobis-2-nitro benzoic acid) were purchased from CDH, New Delhi, India.

C. Experimental animals

Swiss albino mice and wistar albino rats were purchased and housed in Bilwal Medchem and Research Laboratory Pvt. Ltd. Rajasthan, India. Animal were given standard diet and were kept in standard condition of temperature, humidity, light/dark cycle. The protocols for animal experimentation was approved by Institutional Animal Ethics Committee (IAEC), Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) (Reg. No. 2005/PO/RcBT/S/18/CPCSEA).

D. Extraction and GCMS analysis of crude extract

(i) Extraction. 200 g of leaf powder of *Drypetes roxburghii* was defatted using 4000 ml of petroleum ether at 60-80 °C using soxhlet apparatus. It took around 12 cycles for the complete defatting of the leaves. After defatting, the marc obtained was dried till the evaporation of petroleum ether. The dried marc, which weighed around 160 gm, was further used for extraction with 70% ethanol using soxhlet assembly. The extraction was carried out at 65–80°C and continued for the next 8 cycles, i.e., till the solvent in the syphon tube became colorless. Afterward, the extract was collected and the solvent was evaporated using a rotary evaporator under reduced pressure. The crude extract thus obtained was placed in the refrigerator until further use.

(ii) GCMS analysis of crude extract. GC-MS analysis of the above extract was performed using the equipment GC-MS QP-2010 Ultra with Thermal Desorption System TD 20. GC-MS operating parameters were as follows: split Injection mode (split ratio 10.0); column oven tempersture:80° C; injection temperature: 260° C; flow control mode: Linear velocity (40.5 cm/sec); pressure 71.7 kPa; total flow: 16.3 mL/min; column flow: 1.21 mL/min; Purge flow: 3.0 mL/min; ion source temperature- 230° C; interface temperature – 270 °C; solvent cut time: 2.50 min; MS start time - 3.00 min; MS end time: 42.32 min; scan speed: 3333; start m/z: 40.00; end m/z: 650. Helium was used as carrier gas. The components were identified based on Wiley 8 and NIST 1 (The National Institute of Standards and Technology-1) library.

E. Biological activity

(i) Acute oral toxicity study. The acute oral toxicity was performed using the fixed-dose method as per The Organization for Economic Co-operation and Development (OECD) guideline 420 for testing of chemicals. Overnight fasted female Swiss albino mice were administered with a single dose of 2000 mg/kg BW extract. The animals were observed initially for 30 min, then after 4 hours, and then once daily for 14 days for any sign of toxicity (Sireeratawong *et al.,* 2016).

(ii) Anti-epileptic activity. Experimental design: Four groups of Swiss albino mice (25-30 g), each group consisting of six mice of either sex was used for study. Group I received 1% Carboxy methyl cellulose (CMC), p.o and served as control group; group II received diazepam (2.0 mg/kg, i.p) and served as standard group; group III and IV received *Drypetes roxburghii* extract of dose 100 and 200 mg/kg, p.o and named as *Drypetes roxburghii* low dose (DRLD: 100 mg/kg) and *Drypetes roxburghii* high dose (DRHD: 200 mg/kg, p.o) and served as test groups.

Pentylenetetrazol (PTZ) Induced Kindling Epilepsy. The mice received their respective treatments for three days. On the last day of the treatment, 1 h after drug administration, PTZ (80 mg/kg, i.p) was administered to mice of each group. After PTZ administration, the mice were kept in their respective cages and were

observed for convulsive behaviour, initially for 30 min and later for up to 24 h. The time of onset and duration of epilepsy were recorded (Kala *et al*., 2021).

Maximal electroshock seizures. Swiss albino mice received their respective treatment for 3 days. 1 h after the administration of the last dose, the epilepsy was induced using an ear clip electrode to deliver maximal electroshock using an electroconvulsiometer. A current of 150 mA was delivered for 0.2 s. The parameters evaluated were duration of hind limb flexion, hind limb extensor, stupor, and survival/death. The percentage of protection from epilepsy was calculated (Kala *et al*., 2021; Adikay and Govindu 2014).

(iii) Anti-anxiety activity. Experimental design: Swiss albino mice were divided into four experimental group containing six mice in each group. Group I served as control and received 1% CMC; group II served as standard and received Diazepam (2 mg/kg, i.p); group III and IV served as test group and received DRLD (100 mg/kg, p.o) and DRHD (200 mg/kg, p.o).

Light and dark test: The light and dark test is used to evaluate the unconditioned anxiety response in rodents. The apparatus contains two compartments with an opening in between them to allow the movement of rodents between both compartments. The mice were treated with their respective drugs, and one hour after the drug administration, the mice were placed at the centre of the light compartment one by one and allowed to explore the area for 5 minutes. The percentage of time spent in the light area was calculated. With an effective anxiolytic agent, the rodents have a tendency to spend more of their time in the light area, and vice-versa is true for anxiogenic agents (Gabriel de Oliveira *et al*., 2022).

Hole board test. The Hole board test evaluates the exploratory behaviour of the rodents as exposure of the rodents to a new environment induces fear and curiosity in them, which is seen as head dip in the hole board. The apparatus consists of a wooden arena of dimensions of 40 × 40 × 30 cm with twenty holes of 4 cm in diameter placed equidistant to each other. The experimental mice were given their respective treatments and 30 min after the drug administration, the mice were located at the centre of the apparatus and were allowed to explore the area. The number of head dips made by the mice of each group was recorded for 5 min (Asif *et al.,* 2021).

Elevated plus maze. The test is based on the principle that an effective anxiolytic will reduce anxiety in the rodents and increase the time and frequency of stays in open arms (open arm exploration). An elevated plus maze is a wooden apparatus with two open and two closed arms of a height of 40 cm, placed opposite to each other with a central platform at the cross-section of four arms to a height of 50 cm above the floor. The mice were administered with their respective treatments and, after 30 min of drug administration; the mice were placed individually on the central platform with their faces directed toward the enclosed arm. Mice were observed individually for 3 min and time spent in open arms and closed arms; and the numbers of open arm entries were recorded (Asif *et al.,* 2021; Vogel, 2007).

(iv) Anti-depressant activity. Experimental design: Swiss albino mice were divided into four experimental group containing six mice in each group. Group I served as control and received 1% CMC; group II served as standard and received Flouxetine (10 mg/kg, p.o); group III and IV served as test group and received DRLD (100 mg/kg, p.o) and DRHD (200 mg/kg, p.o). Each mice received their respective treatment for 7 days.

Tail suspension test. The mice received their respective treatments as per the protocol mentioned above. One hour after the administration of the last dose, the mice were suspended from 1 cm above the tip of their tail to the edge of the shelf positioned at 55 cm above the platform. The mice were then observed for 5 min, and the time taken for the mice to become immobile for at least 1 min was recorded (Asif *et al.,* 2021; Küpeli Akkol *et al*., 2019).

Monoamine oxidase (MAO)-A and MAO-B inhibitory activity. Preparation of Mitochondria**:** The whole brain of the three mice of each experimental group were isolated after euthanizing them. The brain was sectioned and washed with 0.25 M of sucrose, 0.1 M Tris, and 0.02 M EDTA (pH=7.4). The content was homogenized in a tissue homogenizer for 1 min. After homogenization, the content was centrifuged at 3500 rpm for 10 min. The supernatant thus obtained was collected and again centrifuged for 10 min at 12000 rpm. The precipitate was washed with 10 ml of Sucrose-tris-EDTA and resuspended in 5 ml of the medium. The resulting content is mitochondrial suspension and was stored for 48 h at -18°C. After that, the mitochondrial suspension was taken out of the freezer and mixed with 5 ml of sucrose-tris-EDTA medium. The mixture was then homogenized and spun at 12000 rpm for 20 minutes. The pellet thus obtained was suspended in 50 mM of phosphate buffer (pH 7.4). This was used for the MAO assay (Schurr and Livne 1976).

Lowry method was used to estimate the final protein concentration in the mitochondrial mixture. The MAO assay mixture of 1 ml consisted of 5-HT (4 mM) and beta-phenylalanine (2 mM) as substrates for MAO-A and MAO-B, respectively, 250 µl of mitochondrial fraction, and sodium phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated for 20 min at 37 °C for the reaction to proceed and was halted by the addition of 200 µl of 1 M HCl. The reaction mixture was added with 5 ml of butyl acetate to extract MAO-A and 5 ml of cyclohexane to extract MAO-B. The absorbance was read at 280 nm and 242 nm for MAO-A and MAO-B, respectively (Yu *et al.,* 2002).

(v) Muscle relaxant activity. Experimental design: Swiss albino mice were divided into four experimental group containing six mice in each group. Group I served as control and received 1% CMC; group II served as standard and received Diazepam (2 mg/kg, i.p); group III and IV served as test group and received

DRLD (100 mg/kg, p.o) and DRHD (200 mg/kg, p.o).

Rota rod. The Rota rod apparatus consisted of a rotating iron bar of 7 cm in diameter, positioned 24 cm above the floor at a speed of 12 revolutions per minute (rpm). The mice were initially trained to stay on the rotating rod before conducting the main experiment. On the next day, 30 min before and after the administration of respective treatments, mice of each experimental group were placed individually on a rotating rod and the fall of time was recorded (Asif *et al.,* 2021; Vogel, 2007).

Grip strength test. A horizontal metal wire, about 30 cm above the ground, was suspended. The test animals were placed on it, and as is their natural tendency, they tended to grab it with their forepaws immediately. The metal wire was left hanging open for the mice. Within 5 seconds, a normal mouse would grab and climb the metal wire. Mice that meet this requirement were chosen for the investigation. Following receiving their respective treatments, the mice in each experimental group were individually placed on the suspended metal wire. Each mouse's fall-off time was noted 30 minutes before and after the medication administration (Asif *et al.,* 2021; Vogel, 2007).

Chimney test. The apparatus consisted of a 30 cm long pyrex glass cylinder with a mark made at 20 cm from its base. The mice were given their respective treatments and, 30 min after their drug administration, the mice were individually introduced at one end of the horizontally placed cylinder with their heads directed forward. As the mouse reached another end, the cylinder was positioned vertically. As a general tendency, the mouse tended to climb backward immediately by performing coordinated movements. The time taken by the mouse to perform such a movement was noted down (Asif *et al.,* 2021; Vogel, 2007).

(vi) Anti-oxidant activity. Experimental design: Rats were divided into 5 experimental groups. Group I received vehicle (1% CMC) and served as control group; Group II received Dichlorvos (8.8 mg/kg BW) and served as disease control; Group III received Quercitin (30 mg/kg, BW) and served as standard group; group IV and V received DRLD and DRHD respectively and served as test groups.

Preparation of brain homogenate: The rats were euthanized by cervical dislocation after mild chloroform inhalation. The brains were isolated and put in a chilled 0.9 % NaCl solution. The brain was then homogenised in a tissue homogenizer in 10% of 30 mM Tris-HCl (pH 7.4). The homogenates were centrifuged at 3000 rpm for 10 min.

Estimation of catalase activity. The determination of catalase activity is based on determining the decomposition rate of hydrogen peroxide (substrate of catalase) using a spectrophotometer. For this, brain tissue homogenates were mixed with potassium dichromate and acetic acid (1:3) in boiling water for 10 min, and absorbance was measured at 570 nm (Singh *et al.,* 2018).

Estimation of Malondialdehyde (MDA). After adding 0.5 of the supernatants to 0.5 ml of tris-HCl, the mixture was incubated for two hours at 37°C. After that, 1 ml of 10% tricholoacetic acid was added, and the mixture was centrifuged for 10 minutes at 3000 rpm. After that, 1 mL of 0.67 percent thiobarbituric acid was added. The tubes were placed in boiling water for 10 min. The tubes were taken out and allowed to cool for 10 minutes. In addition, 1 ml of double-distilled water was added before measuring absorbance at 532 nm. The amount of MDA present was given as nmol MDA/mg of protein (Bhosale *et al.,* 2014).

Estimation of Glutathione levels. A millilitre of supernatant was mixed with 1ml of 4% s ulphosalicylic acid. The samples were stored at 4°C. 0.1 ml of supernatant, 2.7 ml of 0.1 M phosphate buffer (pH 7.4) and 0.2 ml of DTNB (5,5, dithiobis-2-nitro benzoic acid) were added to make 3 ml of the reaction mixture. The absorbance was measured at 412 nm. GSH was expressed as ng/mg of protein (Bhosale *et al.,* 2014).

Estimation of nitrite level. The nitrite level, which is a result of the production of nitric oxide (NO) as a result of oxidative stress, was determined spectrophotometrically by mixing brain homogenate with Griess reagent (0.1% N-1-naphthyl ethylene amine dihydrochloride, 1% sulphanilamide, and 2.5% phosphoric acid). The reaction mixture was incubated for 10 min, and the absorbance was taken at 546 nm (Aslam *et al.,* 2021; Bais *et al.,* 2015).

E. Statistical analysis

Graph pas prism version 9 was used for statistical analysis. The data is expressed as Mean ±SEM and analyzed using one-way ANOVA followed by Dunnett's test of multiple comparisons. The mean of each treatment group was compared with the mean of control or disease control group. The criteria for statistical significance were $p<0.05$.

RESULTS AND DISCUSSION

Neural problems have traditionally been treated with herbal medication. While the exact mechanisms of action of herbal medications are still unknown, several of them have been demonstrated to have anti-inflammatory and/or antioxidant effects (Kumar and Khanum 2012). *Drypetes roxburghii* Wall. has historically been credited with playing a significant part in the old Eastern Ayurvedic and Unani systems of holistic treatment. In several conventional medical systems, *Drypetes roxburghii* Wall. leaves are said to have beneficial therapeutic properties (Minj and Britto 2018).

To the best of our knowledge, no scientific study has been conducted which demonstrates the neuroprotective effects of the leaves of *Drypetes roxburghii*. Therefore, the present study was aimed at analyzing the

neuroprotective potential of the leaves of *Drypetes roxburghii.*

A. GC-MS analysis of crude extract of Drypetes roxburghii leaves

The crude extract of leaves of *Drypetes roxburghii* was obtained and the phytoconstituents was identified using GC-MS analysis. Table 1 and Fig. 1 presents the phytoconstituents present in the *Drypetes roxburghii* extract. The GC-MS analysis of *Drypetes roxburghii* extract revealed the presence of Butanedinitrile-2,3-dimethyl- (0.75%), n-Propyl heptyl ether (13.76%), n-Nonyl-cyclopropane (1.36%), 2-Ethyl-2-methylbutanoic acid (0.50%) , Thiirane, methyl- (0.57%), 5-Ethylhydantoin (1.99%), 1-Tetradecene (2.71%), 1,3-Oxazinane-2-thione (5.06%), Phenol,2,4-Bis (1,1-dimethylethyl) (2.90%), 5,6-Dimethltetrahydro-1,3-oxazine-thione (6.48%), 9-Eicosene (3.58), 1-Heneicosanol (4.01%), Pentadecanoic acid (4.86%), 1-Heneicosanol (3.97%), Cis-Vaccenic acid (21.77%), 9-Octadecenoic acid (2.10%), n-Tetracosanol-1 (3.15%), Eicosyl trifluoroacetate (2.50 %) , 1,2-Bezenedicarboxylic acid (14.81%), Eicosyl heptafluorobutyrate (1.49%), Tetracontane (0.26%), Eicosyl heptafluorobutyrate (0.90%), Tetracontane (0.53%). Among the above mentioned phytoconstituents

Cis-Vaccenic acid (21.77%), 1,2-Bezenedicarboxylic acid (14.81%), n-Propyl heptyl ether (13.76%), 1,3-Oxazinane-2-thione (5.06%) in maximum amount. Also, the extract contains 5-Ethylhydantoin (1.99%).

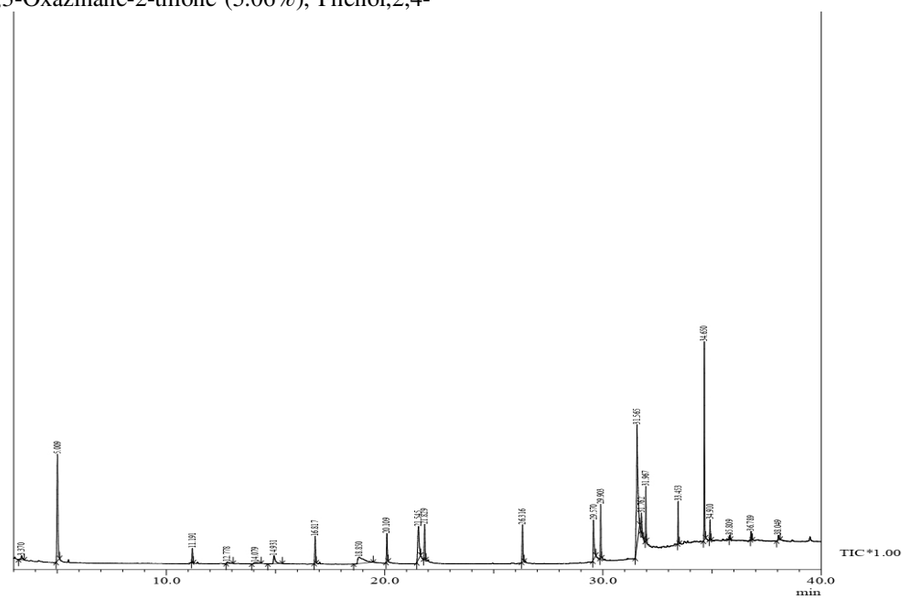

Fig. 1. GCMS chromatogram of crude extract of *D. roxburghii* leaves.

Table 1: Phytochemical composition of leaves extract of *Drypetes roxburghii.*

Peak	Name of the compound	Retention time	% Area
1	Butanedinitrile-2,3-dimethyl-	3.370	0.75
2	n-Propyl heptyl ether	5.009	13.76
3	n-Nonyl-cyclopropane	11.191	1.36
4	2-Ethyl-2-methylbutanoic acid	12.778	0.50
5	Thiirane, methyl-	14.079	0.57
6	5-Ethylhydantoin	14.931	1.99
7	1-Tetradecene	16.817	2.71
8	1,3-Oxazinane-2-thione	18.850	5.06
9	Phenol, 2,4-Bis (1,1-dimethylethyl)	20.109	2.90
10	5,6-Dimethltetrahydro-1, 3-oxazine-thione	21.545	6.48
11	9-Eicosene	21.829	3.58
12	1-Heneicosanol	26.316	4.01
13	Pentadecanoic acid	29.570	4.86
14	1-Heneicosanol	29.903	3.97
15	Cis-Vaccenic acid	31.565	21.77
16	9-Octadecenoic acid	31.762	2.10
16	n-Tetracosanol-1	31.967	3.15
17	Eicosyl trifluoroacetate	33.453	2.50
18	1,2-Bezenedicarboxylic acid	34.650	14.81
19	Eicosyl heptafluorobutyrate	34.910	1.49
20	Tetracontane	35.809	0.26
21	Eicosyl heptafluorobutyrate	36.789	0.90
22	Tetracontane	38.049	0.53

B. Acute oral toxicity study

The extract was checked for its toxicity profile using an acute oral toxicity study based on two doses, 100 and 200 mg/kg BW. The test extract was further assessed for various neuroprotective effects such as anti-epileptic activity, anti-anxiety activity, anti-depressant, muscle relaxant, and anti-oxidant.

C. Anti-epileptic activity

Anti-epileptic activity of *Drypetes roxburghii* extract was assessed using PTZ induced epilepsy and MES induced convulsion.

Pentylenetetrazol (PTZ) Induced Epilepsy. As shown in Fig. 2 (A) there is no significant decrease in the onset of convulsion in mice treated with DRLD and DRHD compared to the mice in the control group. On the other hand, in mice of the standard group treated with diazepam, convulsions were not observed. Additionally, Diazepam, DRHD and DRLD significantly reduced the duration of PTZ-induced convulsion compared to the control group at $p<0.001$. The onset and duration of convulsion were compared between the standard group and the test groups. The difference in reducing the duration of convulsions (Fig. 2B) was found to be significant between the diazepam treated group and the DRHD treated group at $p<0.01$; and between the diazepam treated group and the DRLD treated group at $p<0.0001$. This means that the activity of DRHD is closer to standard diazepam.

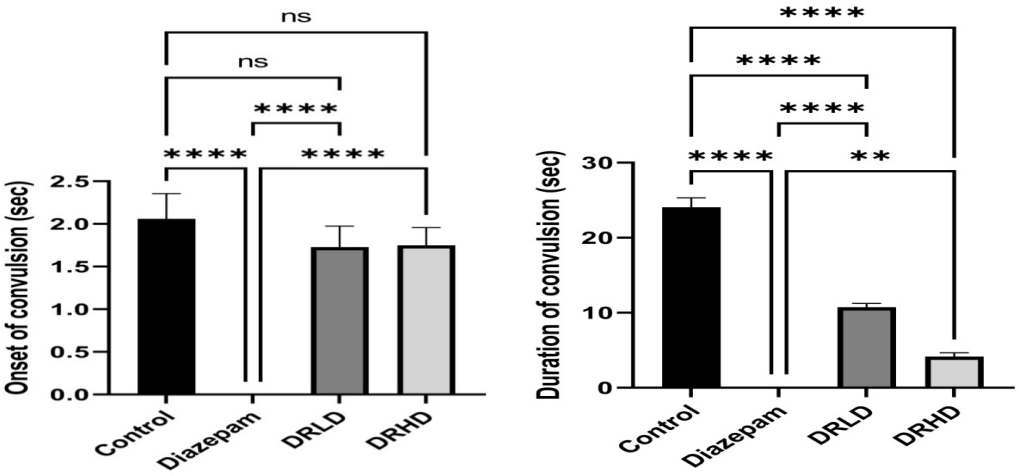

Fig. 2. Effect of various treatments on onset of convulsion against PTZ induced convulsion (A); Effect of various treatments on duration of convulsion (sec) against PTZ induced convulsion (B).

Pentylenetetrazole (PTZ) is a common medication used to cause seizures. It functions as an antagonist of GABA-A receptors. PTZ increases neuronal activity by suppressing the action of inhibitory synapses. An acute seizure is also brought on by a single injection of a convulsive dosage of PTZ (Shimada and Yamagata 2018). The test extract was able to reduce the duration of seizures. One of the reasons may be the presence of 5-Ethyl hydantoin in the hydroalcoholic extract of *Drypetes roxburghii*. The anticonvulsant effect of hydantoin derivatives is a result of their ability to selectively inhibit high-frequency neuronal activity. Their binding to the voltage-sensitive sodium channels responsible for the action potential is the underlying molecular process (Trišović *et al.,* 2011). Therefore, the antiepileptic effect of *Drypetes roxburghii* extract may be attributed to the presence of hydantoin moiety in it.

Maximal electroshock seizures. As shown in Fig. 3, mice treated with Diazepam 2 mg/kg, i.p did not show any flexion or extension and the duration of stupor was reduced significantly compared to mice in the control group at $p<0.0001$. Treatment with DRLD and DRHD reduced the duration of extension and stupor compared to the mice in the control group at $p<0.0001$. DRHD reduced the duration of flexion compared to the control group at $p<0.001$ whereas DRLD did not reduce the duration of flexion compared to the control group. Test groups (DRLD and DRHD) were also compared with the standard group (Diazepam). The effect of DRHD in reduction of stupor is comparable to that of standards.

Most of the time, the MES epilepsy test is used to see how well antiepileptic drugs work against a generalized tonic-clonic seizures (Nieoczym *et al.,* 2018). It is known to activate the sodium channel and increase sodium uptake. Additionally, it increases the levels of the excitatory neurotransmitter glutamate, which is known to connect with NMDA receptors and induce petit mal epilepsy in people. In the above investigation, both doses of *Drypetes roxburghii* reduced the duration of flexion, extension, and stupor (Viswanatha *et al.,* 2016). Similar to phenytoin, 5-ethyl Hydantion is an imidazole derivative chemical found in *D. roxburghii*. Anticonvulsant activity of hydantoin-containing medicines, such as Alkoxymethyl, acyloxymethyl, and mixed alkylalkoxymethyl or alkylacyloxymethyl derivatives of 5-ethyl-5-phenylhydantoin, has been demonstrated against maximal electroshock epilepsy and PTZ-induced kindling epilepsy. The pharmacology of hydantoin is to decrease synaptic transmission (Cook *et al.,* 2011). Based on the result from PTZ and MES induced seizures, it can be suggested that *D. roxburghii* hydroalcoholic extract exert anti-epileptic effect attributed to the presence of hydantoin moiety in it.

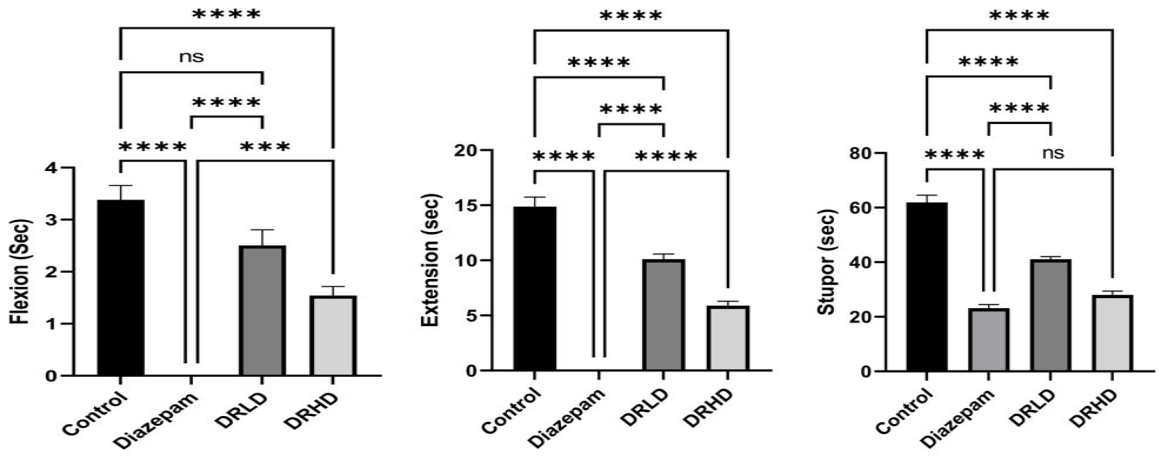

Fig. 3. Effect of various treatments on Flexion (A); Extension (B), and stupor (C) against Maximal electroshock seizures.

D. Anti-anxiety activity

(i) Light and dark model. As shown in Fig. 4, the mice of the control group showed a lower percent of time spent in light areas (53 ± 1%). Treatment with diazepam and DRHD increased the time spent in the light area to 83 ±3.94 % and 79 ± 1.38 % respectively, compared to the control group at p<0.0001. Additionally, treatment with DRLD increased the time spent in light areas to 69 ± 0.65 % compared to control group at p<0.001. The results of the test groups were compared with those of standard diazepam. No significant difference in the activity was found between DRHD and diazepam, indicating that the effect of DRHD was close to that of diazepam.

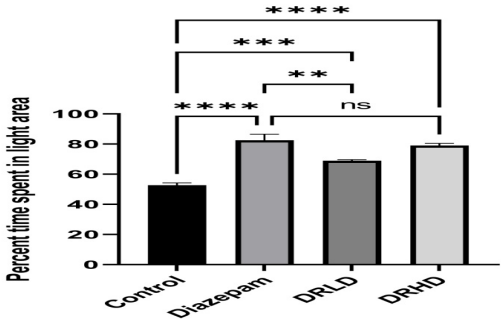

Fig. 4. Effect of various treatments on percentage of time spent in light area in light and dark animal model.

The light-dark (LD) test is frequently used to detect anxiety-like behaviour in adult rats and is based on an approach-avoidance conflict. Anxiolytic medications enhance the number of compartment crossings, according to Crawley and colleagues (Arrant *et al.,* 2013). Shorter entry latencies and/or more time on the light side of the box indicate reduced anxiety or an anxiolytic-like effect (Lezak *et al.,* 2017).

Our results show that our extract at both doses increased the percent time spent in the light area compared to the control group. However, the effect of a larger dose of 200 mg/kg was found to be comparable with the standard.

(ii) Hole board test. As shown in Fig. 5, the mice in the control group showed the least count of head dip in hole board apparatus, i.e. 19.5 ± 1.17. Treatment with Diazepam and DRHD significantly increased the number of head dip counts to 45.16± 2.38 and 37 ± 1.032 respectively, compared to mice in the control group at p<0.0001. Administration of DRLD also increased the number of head dip counts to 28.17 ± 1.53 compared to the control group at p<0.01. On comparing test groups with the standard group, the activity of DRHD was closer to that of standard diazepam.

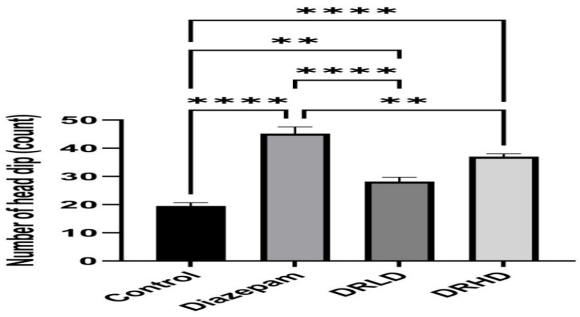

Fig. 5. Effect of various treatments on the number of head dip by the Experimental mice in hole board test.

Boissier and Simon invented the hole-board test in (1962 and 1964) to measure an animal's response to a new environment. The hole-board test has been used to measure animal emotions, anxiety, and stress. This test allows for a thorough description of animal behaviour because various behaviors may be easily seen and quantified. Changes in head-dipping behaviour may represent animals' anxiogenic and/or anxiolytic states (Takeda *et al.,* 1998). The test drug was able to increase the head dipping behavior in hole board test depicting its anxiolytic property.

Elevated plus maze. As shown in Fig. 6A, the mice in the control group showed the least number of open arm entries (4.34 ± 0.67). Treatment with diazepam and DRHD significantly increased the count of open arm entries to 13.34 ± 0.88 and 13 ± 0.97 respectively, at p<0.0001. However, treatment with DRLD does not show any significant difference in increasing the number of open arm entries. On comparing the results

of test groups with diazepam, no significant difference in the activity was observed between diazepam and DRHD, which suggests that these two treatments have comparable activity. On the other hand, the effect of DRLD in enhancing the number of open arm entries was very small, with a significant difference at $p<0.0001$.

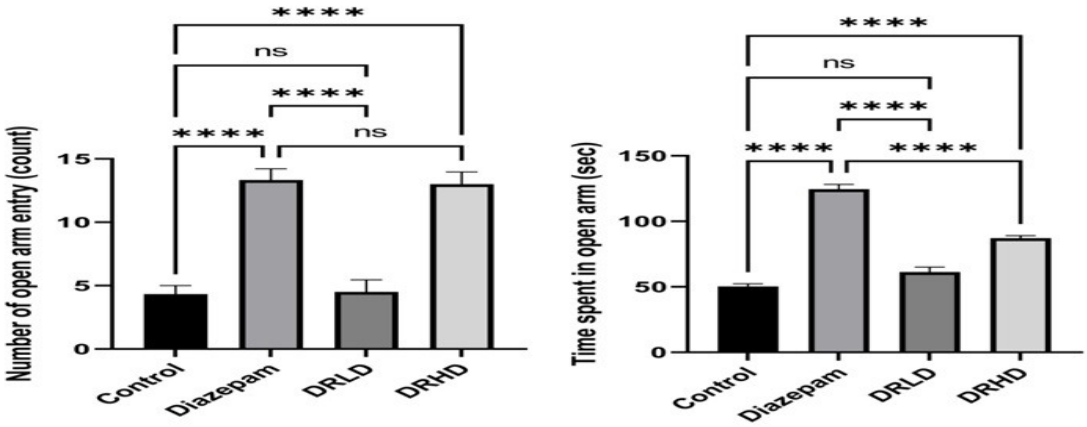

Fig. 6. Effect of various treatment on (A) number of open arm entry (count) and (B) time spent in open arm (sec).

Similarly, in Fig. 6B diazepam and DRHD significantly increased the time spent in the open arm to 124.5 ± 3.70 sec and 87.17 ± 1.95 sec compared to the control group with time spent in the open arm of 50.34 ± 2.011 sec, respectively at $p<0.0001$. No significant difference was found in time spent in the open arm between the control group and the DRLD group. On comparing the results of test groups with diazepam, a significant difference was found between diazepam and DRHD; diazepam and DRLD in time spent in the open arm, suggesting the enhanced potency of diazepam compared to test groups. However, the activity of DRHD was closer to diazepam.

Elevated plus maze is responsive to anxiolytic and anxiogenic agents acting on the GABAA-BZD receptor. Elevated Plus-Maze is an established model for testing sedative and anxiolytic medicines like BZDs. Anxiolytics improve open arm exploration, and anxiogenics decrease it (Asif *et al*., 2021).

The test drug at 200 mg/kg increased the number of open arm entries and time spent in open arms, depicting the anxiolytic effect of the test drug. In a study, a mixture of fatty acids such as lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, elaidic acid, and linoleic acid were assessed for their anxiolytic activity in Wistar rats on postnatal day. The results showed that these fatty acids affected GABAergic neurotransmission in 28-day-old rats in a way that made them act anxious (Bernal-Morales *et al.,* 2017). The test extract was also found to contain several fatty acids such as pentadecanoic acid (4.86), Cis-Vaccenic acid (21.77%), and 9-Octadecenoic acid which may be responsible for the anxiolytic effect of the extract through modulating GABAergic neurotransmission.

E. Anti-depressant activity

(i) Tail suspension test. As shown in Fig. 7, only the group of mice treated with fluoxetine was able to reduce the duration of immobility to 61.5 ± 3.17 sec compared to mice of the control group at 142.67 ± 4.12 sec at $p<0.0001$. However, treatment with DRLD and DRHD does not reduce the duration of immobility compared to the control group. The tail suspension test is a behavioral technique that is frequently used to examine a drug's potential antidepressant effects. The mice are suspended from the floor by their tails during this test, and while the behaviour of the animal in response to these difficult conditions is studied throughout, the mouse eventually becomes immobilized and in distress due to the test's failure. The extent of immobility is thought to be a representation of depression in the mice, and a potent antidepressant must be able to shorten the period of immobility. The tail suspension test is preferred to the forced swim test because it doesn't result in hypothermia like the latter does. Additionally, it is employed to assess the impact of ecological, neurological, and genetic factors (Takeda *et al.,* 1998). The test extracts do not reduce the duration of immobility compared to the control.

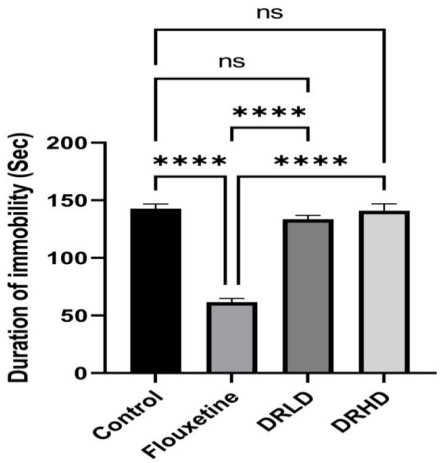

Fig. 7. Effect of various treatments on mice's duration of immobility (sec) in tail suspension test.

(ii) MAO-A and MAO-B inhibition. As shown in Fig. 8A and 8B, among all the treatments, *i.e.,* flouxetine, DRLD and DRHD; only flouxetine inhibited MAO-A and MAO-B compared to mice of control group at $p<0.01$ and $p<0.001$. However, none of the test groups *i.e*. DRLD and DRHD inhibited MAO-A and MAO-B significantly compared to the control group. Also, the percentage of MAO-A and MAO-B inhibition with flouxetine was 49.14 and 44.83 % respectively; for DRHD, the percentage of MAO-A and MAO-B inhibition was quite low, *i.e.* 5.51 and 1.84 respectively; for DRLD, the percentage of MAO-A and MAO-B inhibition was found to be 8.54 and 1.73. Based on the tail suspension test and inhibition of MAO-A and MAO-B, it is seen that the test groups do not show protective activity against depression. One of the recognized antidepressant mechanisms is MAO-A inhibition. Both reversible and irreversible MAO-A inhibitors are frequently used as antidepressants. Neurotransmitters, xenobiotics, and endogenous amines like serotonin, dopamine, and noradrenaline are all metabolized by MAO. MAO-A inhibitors function as powerful antidepressants. Therefore, it is possible that MAO inhibition provides oxidative stress protection. According to reports, phytoconstituents may inhibit MAO, which would have neuroprotective and anti-depressive effects (Herraiz *et al*., 2018). However, in our study the test extracts do not inhibit MAO-A and MAO-B. Based on the results of the above two models, it can be said that the test extracts do not exert an anti-depressant effect.

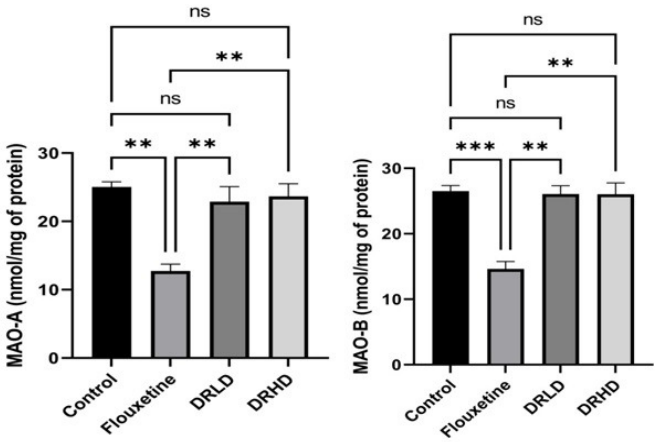

Fig. 8A and B. Effect of various treatments on inhibition of MAO-A and MAO-B enzymes.

F. Muscle relaxant activity

(i) Rota rod test. As shown in Fig. 9, treatment of mice with Diazepam, DRHD, and DRLD significantly reduced the fall-off time of the rota rod to 10.31 ± 1.38 sec, 43.27 ± 3.98 sec, and 32.41 ± 1.45 sec respectively compared to control group with a fall-off time of 86.27 ± 2.54 sec at $p<0.0001$. On comparing the fall-off time between diazepam and test groups, the activity of the test groups in reducing the fall-off time is significantly different from diazepam at $p<0.0001$ suggesting that diazepam is more potent in relaxing the skeletal muscles compared to the test drugs.

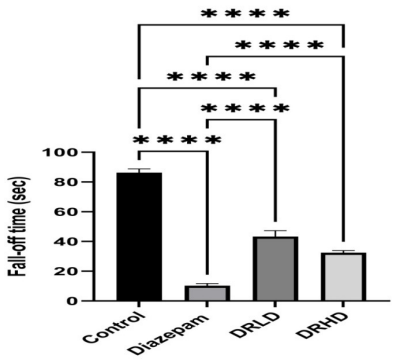

Fig. 9. Effect of various treatments on fall-off time (sec) of mice using rota rod.

(ii) Grip strength test. As shown in Fig. 10, only in diazepam treated mice skeletal muscle was relaxed as there was significant decrease in fall-off time from the suspended metal wire compared to control group at $P<0.01$. Both the test group does not show any significant reduction if fall-off time compared to control group.

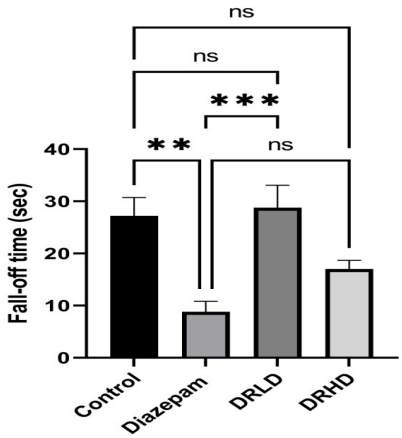

Fig. 10. Effect of various treatments on fall-off time (sec) of mice using grip strength test.

As shown in Fig. 11, diazepam and DRHD treated mice taken greater time to leave the cylinder *viz.*, 36.34 ± 3.60 sec and 22.34 ± 1.47 sec respectively compared to the control group with 3.84 ± 0.60 sec to leave the cylinder at p<0.0001. However, DRLD treated mice does not show any significant difference in time taken to leave the cylinder (6.84 ± 1.16 sec). On comparing the activity of test groups with standard diazepam, there was less significant difference in activity between diazepam and DRHD suggesting that the effect of DRHD is closer to that of diazepam.

For assessment of muscle relaxant activity, the rota rod test, grip strength test, and chimney test were used. The Rota Rod Test measures the rodent's ability to maintain its position on the revolving rod in order to determine if medications have the potential to impair motor coordination. When 50% of the rats are unable to remain on the revolving rod, it is the test's endpoint (George *et al*., 2012). The grip strength test is performed to evaluate neuromuscular function and muscular strength in rats that are known to be affected by sedatives, muscle relaxants, and toxins (Vogel, 2007). In our study, the fall-off time of the test group animals from rota rod, metal wire thread, and cylinder was significantly reduced compared to control, indicating its muscle relaxant activity.

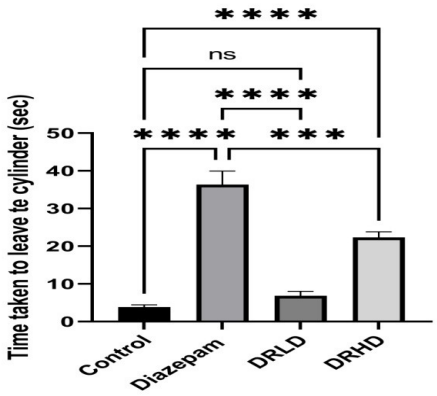

Fig. 11. Effect of various treatments on time taken by the mice to leave the cylinder using chimney test.

The muscle relaxant activity of the extract may be attributed to 5-Ethyl hydantoin. A hydantoin-based substance called dandrolene is used to treat malignant hyperthermia by relaxing the skeletal muscles. This 1-aminohydantoin, which is a structural counterpart of nitrofurantoin, has been reported on several occasions during the last ten years. In 1979, Norwich Eaton began marketing it under the brand name Dantrium. It is used to relax muscles and stop malignant hyperthermia. 3-[[5-(p-Nitrophenyl) furfurylidene]amino] Hydantoin, on the other hand, had the ability to relax skeletal muscles (Konnert *et al*., 2017; Schwan and Ellis 1975).

Dantrolene acts by reducing the actin-myosin excitation-contraction coupling interaction inside the individual sarcomere in skeletal muscle via intracellular action. The sarcoplasmic reticulum's ryanodine receptors are antagonised in order to prevent the release of calcium ions, which are necessary for the contraction process, hence relaxing the muscle. The same may be true for the extract relaxing the skeletal muscle (Ratto and Joyner 2022).

G. Anti-oxidant activity

In our study, we used Dichlorvos to induce oxidative stress in rats and observed the levels of CAT, GSH, MDA, and nitrite (Nazıroğlu, 2012) It is thought that pesticides such as Dichlorvas damage cells' lipoidal matrix, causing reactive oxygen species (ROS) and oxidative stress. Proteins, DNA, and lipids may be oxidatively modified as a result of excessive ROS generation. The detoxification of these ROS and cell protection are provided by endogenous non-enzymatic (glutathione, GSH) and enzymatic (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST)) antioxidants. Continued exposure to these pesticides also decreases these endogenous antioxidants (Singh *et al*., 2012).

(i) Brain catalase level. As shown in Fig. 12, the catalase level was found to be at a minimum (40.167 ± 3.178 nm/min/mg of protein) in the brains of disease control group animals. Treatment with Quercetin 30 mg/kg, DRLD, and DRHD significantly elevated brain catalase levels to 134.67 ± 3.37, 62.59 ± 2.88, and 86.834 ± 3.43 nm/min/mg of protein, respectively at p<0.0001. A typical antioxidant enzyme called catalase is present in almost all living things that are exposed to oxygen. H_2O_2 is reduced to water by the action of catalase. Organic hydroperoxides may also be eliminated by catalase. Catalase also oxidizes toxins such phenols, formic acid, formaldehyde, and alcohols using H_2O_2 (Nazıroğlu, 2012).

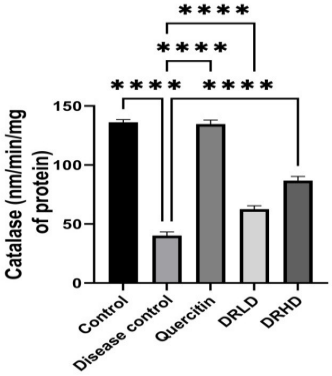

Fig. 12. Effect of various extract on brain catalase level of experimental animal.

(ii) Brain MDA level. As shown in Fig. 13, the disease control rats showed a maximum brain MDA level of 59.435 ± 1.24 nm/mg of protein. Treatment with quercetin and DRHD lowered the brain MDA level to 20.53 ± 1.10 and 35.12 ± 2.54 nm/mg of protein compared to disease control at p<0.0001. Also, DRLD treatment lowered the brain MDA level to 48.045 ± 2.14 nm/mg of protein compared to disease control at p<0.001. Many studies have shown that oxidation and glycation stress are major causes of age-related chronic diseases and neurodegenerative diseases. These stresses lead to the production of toxic intermediates, mostly unsaturated carbonyls like malondialdehyde (MDA) and formaldehyde (FA), which are a major cause of the

biochemical changes associated with ageing in both animals and humans, such as brain degeneration (Li *et al*., 2010).

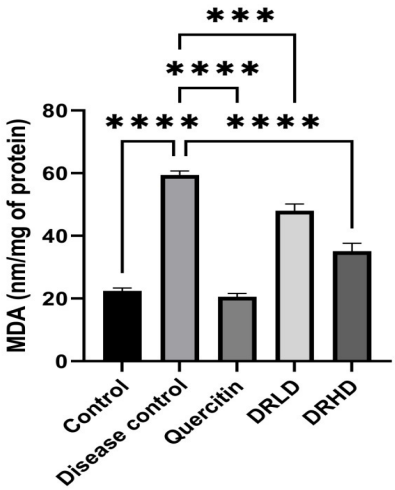

Fig. 13. Effect of various treatments on brain MDA level of experimental animals.

As shown in Fig. 14, brain GSH level was found to be lowest (6.74 ± 0.98 ng/mg of protein) in disease control animal. Treatment with quercitin significantly enhanced the brain GSH level to 16.12 ± 1.54 ng/mg of protein compared to disease control at p<0.0001. Furthermore, treatment with DRHD elevated the GSH level to 12.67 ± 1.27 ng/mg of protein compared to disease control at p<0.01. However, DRLD shows an insignificant increase in brain GSH level compared to disease control animals. Glutathione (GSH, -glutamyl-cysteinyl-glycine) is an intracellular linear tripeptide present in all mammalian cells. GSH acts as a potent anti-oxidant by interacting with reactive oxygen species and reactive nitrogen species (ROS/RNS) and detoxifying them. Low GSH levels trigger ROS production, which is linked to cell death and diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (Dwivedi *et al.,* 2020).

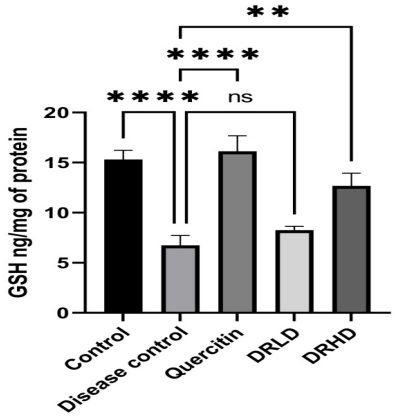

Fig. 14. Effect of various treatments on brain GSH level of experimental animals.

(iii) Brain nitrite level. As shown in Fig. 15, the brain nitrite level was found to be highest (6.79 ± 0.31 (nM/mg of protein) in disease control animals. Treatment with quercitin and DRHD reduced nitrite level to 2.7 ± 0.47 (nM/mg of protein (p<0.0001) and 3.28 ± 0.49 nM/mg of protein (p<0.01) respectively compared to disease control. Treatment with DRLD showed an insignificant reduction in brain nitrite compared to disease control.

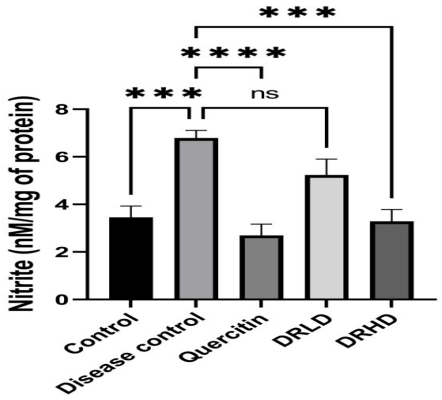

Fig. 15. Effect of various treatments on brain nitrite level of experimental animals.

In the CNS, NO is a highly reactive signal molecule. The substance is a gaseous chemical messenger that influences mediator release, synaptic plasticity, memory formation, receptor function, and intracellular signal transmission. However, when greater fluxes of these mediators are produced, as during the process of excitotoxication, pathogenic symptoms may manifest. Furthermore, NO production results in the formation of O2-, ONOO-, and OH• radicals (Dwivedi *et al.,* 2020).

Therefore, in our study, we estimated the level of these neurochemicals after the administration of dichlorovos and various treatments. The extract exhibited potential anti-oxidant properties as it elevated brain catalase and brain GSH levels and reduced brain nitrite and brain MDA levels significantly compared to disease control. The anti-oxidant property may be attributed to the presence of many anti-oxidant phytoconstituents in the extract, such as phenol, 2, 4-bis (1, 1- dimethylethyl) which is reported to have anti-oxidant, anti-cancer, anti-fungal, and anti-bacterial properties (Jianguo *et al.,* 2019). In another study, the bulb of Allium chinense was found to be rich in tetracontane, eicosane, and 1-heneicosanol and possessed antioxidant scavenging activity (Rhetso *et al.,* 2020).

Therefore, presence of these compounds in the extract may be responsible for its anti-oxidant property

CONCLUSIONS

The hydroalcoholic crude extract of *Drypetes roxburghii* leaves revealed the presence of 22 compounds. When the extract was evaluated for its Neuroprotective activity, it showed significant anti-epileptic, anti-anxiety, muscle relaxant, and anti-oxidant activity. Among the identified compounds 5-Ethyl hydantoin, pentadecanoic acid, Cis-vaccenic acid, 9-octadecenoic acid, and 2, 4-bis (1,1, dimethyl ethyl) are thought to be responsible for the above mentioned activities.

FUTURE SCOPE

Isolation of the compounds present in the crude extract will serve to identify the phytoconstituents responsible for neuroprotective activity.

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