Evaluation Of Neuroprotective Activity In Scopolamine Induced Dementia In WistarRats By Using Various Pharmacological Equipment And Its Histopathology

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Abstract:
Nature is the best source of complementary and alternative medicine. The plant Biophytum reinwardtii has been used traditionally in pain, inflammatory and oxidative stress related disorders. In this consequence, fraction of methanolic extract of Biophytum reinwardtii was selected to explore the ability of this plant to enhance cognitive function, brain antioxidant enzymes and anti-acetyl cholinesterase activity which can be used for the treatment of oxidative stress related disorders like Alzheimer's disease (AD). The purpose of this study was to investigate the neuroprotective effect of HEMBR on learning and memory impairment in scopolamine-induced rats of dementia and oxidative stress. Treatment with HEMBR (i.e., 50 and 100 mg/kg b.w.) was investigated in scopolamine-treated Swiss albino male rats.

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Introduction:

Introduction: Alzheimer's disease has been identified as a protein misfolding disease (proteopathy), caused by plaque accumulation of abnormally folded amyloid beta protein, and tau protein in the brain. Plaques are made up of small peptides, 39–43 amino acids in length, called amyloid beta (Aβ). Aβ is a fragment from the larger amyloid precursor protein (APP). APP is a transmembrane protein that penetrates through the neuron's membrane. APP is critical to neuron growth, survival, and post-injury repair. In Alzheimer's disease, gamma secretase and beta secretase act together in a proteolytic process which causes APP to be divided into smaller fragments. One of these fragments gives rise to fibrils of amyloid beta, which then form clumps that deposit outside neurons in dense formations known assenile plaques. AD is also considered a tauopathy due to abnormal aggregation of tau protein. These microtubules act like tracks, guiding nutrients and molecules from the body of the cell to the ends of the axon and back. A protein called tau stabilizes the microtubules when phosphorylated, and is therefore called a microtubule-associated protein.

In AD, tau undergoes chemical changes, becoming hyper phosphorylated; it then begins to pair with other threads, creating neurofibrillary tangles and disintegrating the neuron's transport system (1-11)

Materials And Methods:

Neuro protective Activity:

Animals used in the study:

Healthy adult albino Wistar rats weighing 200-250grams of wistar rats were selected for the study. Animals were housed in appropriate cages in uniform hygienic conditions and fed with standard pellet diet (Amrul Laboratory ANIMAL Diet) and water ad Libitum. Animal studies had approval of IACE committee for the purpose of control and supervision of experiments on animals (CPCSEA).

Scopolamine induced dementia:

Total 42 rats were taken, each group contains 6rats.

Group I: Received normal saline

Group II: Received Scopolamine 5 mg/kg (i.p.) for 7 days and allowed access to normal food and water.

Group III: Received Scopolamine 5 mg/kg (i.p.). Donepezil (2mg/kg) for 7 days.

Group IV : Received Scopolamine 5 mg/ kg (i.p.). Plant extract (Fraction 1) low dose

Group V: Received Scopolamine 5 mg/ kg (i.p.). Plant extract (Fraction 1) high dose

Group VI: Received Scopolamine 5 mg/ kg (i.p). Plant extract (crude) low dose

Group VII: Received Scopolamine 5 mg/ kg (i.p). Plant extract (crude) high dose

At the end of treatment period, rats underwent behaviour study for seven days to evaluate learning and memory status. At experiment termination, rats were sacrificed and brain tissues were collected. Each brain was divided into halves; right and left half hemisphere. One half was fixed and stored in 10% formaldehyde for histopathological studies. The second half was stored at -80˚C for biochemical investigations.

Chemicals used in study:

DRUGS**:** Scopolamine, Donepezil.

SOLVENTS**:** DMSO, Phosphate buffer, Hexane, Ethyl acetate, Methanol,

REAGENTS**:** Ellman'sreagent, Acetyl thiocholine

Equipment's used in study:

Acto photo meter, Jumping box, UV spectro photometer, Cooling centrifuge, scientific microscope, Rota Vaccume evaporator, Soxhlet apparatus, IR spectrophotometer.

Behavioural parameters:

Locomotor activity test (Reaction time) by Actphoto meter:

The locomotor activity (horizontal activity) can be easily measured using an actophotometer which operates on photoelectric cells which are connected in a circuit with a counter, a count is recorded. An actophotometer could have either circular or square arena in which the animal moves. Both rats & mice may be used for testing in this experiment.

Figure-1: Actophotometer

Jumping Box:

This test was used to evaluate the Nootropic potential of the plant extracts. It comprises of a acrylic shuttle avoidance box (50 X 25 X 25 cms) whose floor is made of a series of 1mm caliber parallel bronze bars divided at the mid line by a 1m high acrylic hurdle. The conditioned stimulus was a 5 second, 70 decibel, 1 kilohertz buzzer. Each sound was immediately followed by an unconditioned stimulus of 2 seconds, 0.5 mili ampere foot shock. Training and test sessions were procedurally identical. Rats are allowed to explore the box freely for 3 to 5 minutes after which they received foot shock trials with an inter trial interval of 10 to 50 seconds. Each animal was subjected to an experimental session of ten cycles

.Each cycle had a total duration of sixty second and started with a conditioning warning stimulus which was terminated by a correct avoidance response i,e electric shock. The aversive stimulus was terminated by a correct escape response i.e., jumping into the other compartment. Rats avoided shocks by crossing the hurdle during the buzzer sound (conditioned response) [12-15].

Figure-2: Jumping Box

Elevated plus maze:

Rats have an aversion for open-high spaces and prefer enclosed arm then the open arm. They spend more time in enclosed arm. Nootropic agents improve the acquisition process in rats subsequent to training session. Rats avoid open arm exploration and prefer enclosed arm The plus maze has two opposite arms 50 X 10 cms, crossed with two enclosed arms of the same dimensions with walls 40 cms high. The arms were connected with a central square 10 X 10 cms, giving the apparatus a plus sign appearance. The maze was elevated at 50cms above the floor in a dimly lit room. Naïve rats were placed individually in the center of the maze facing towards an enclosed arm. Initially they were allowed to explore the maze. During the next 5 minute the number of entries and the time spent on the open arm and the enclosed arm were recorded. An arm entry was defined when all four limbs were on the arm [14].

Figure-3: Elevated plus maze

Neurochemical parameters:

- Assay of acetyl cholinesterase activity by Elman's method
- Assay of catalase activity

Estimation of Acetyl cholinesterase levels:

The assay is based on measurement of the change in absorbance at 412 nm. The assay usesthe thiol ester acetylthiocholine instead of the oxy ester acetylcholine. AChE hydrolyses the acetyl-thiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the dithiobis-nitrobenzoic acid (DTNB) liberating nitro-benzoate, which absorbs at 412 nm. The reaction is shown below:

Reaction:

Acetylthiocholine (enzyme) ⟶ thiocholine + acetate Thiocholine + DTNB ⟶ yellow color

Procedure

The brain cholinesterase activity was measured by the method of Ellman. A 0.4ml aliquot of the prepared homogenate was added to a cuvette containing 2.6ml of sodium phosphate buffer (pH 7.2, 0.1M). To this, 100µl of Ellman's reagent (DTNB) was added and taken into a photocell [55]. The absorbance was set to zero at 412 nm when the fluctuations stopped. Of the substrate (Acetyl thiocholine iodide) 20µl was added. A change in the absorbance per minute was noted.

Calculations

AChE activity is calculated using the following formula

$$R = \frac{\Delta A}{1.36 \times 10^{4} \times \frac{1}{400} \times \frac{X}{(\quad)C}}$$

$$3120 = 5.74\ (10^{-4})^{\Delta A} \quad C0$$

Where,

ΔA = Change in absorbance per minute

C0 = Original concentration of the tissue (mg/ml)

R = Rate in moles substrate hydrolyzed per minute per gram of tissue

Estimation of Catalase activity:

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water andoxygen, using either an iron or manganese cofactor . This protein is localized to peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate and follows a Ping-Pong mechanism. Its cofactor is oxidised by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate. Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase — "acatalasemia" — or mice genetically engineered to lack catalase completely, suffer few ill effects. [15].

Procedure

Catalase is assayed by the method of disappearance of H2O2. The mixture consists of 1.95 ml of phosphate buffer (0.05 M, pH-7), 1 ml of H2O2 (0.019 M) and 0.05 ml sample (10 % w/v) in a final volume of 3 ml. control cuvette contains all the components except substrate. Change in absorbance is then recorded at 240 nm and the catalase activity is calculated.

$$\text{CAT U} = \frac{\text{mg Protein}}{\text{OD240 X VOLUME OF ASSAY}} \times \frac{0.081 \times \text{VOLUME OF ENZYME}}{\text{X mg of protein}}$$

Where;

OD = Optical density at 240 nm

0.081 = Extinction coefficient

Histopathological studies:

Because of the important role of hippocampus in the memory, its histopathological was investigated. So removed rat's hippocampal tissues were fixed in 10% neutral buffered formaldehyde for 24 hours, embedded in paraffin and cut into 3–4 µm thick sections by a microtome (Leica SM2000R, Germany). The tissue sections were de-paraffinised in xylene. The slides were stained with Hematoxylin and E o s i n (H&E) according to the procedure of Wilson et al. and viewed under a light microscope (Labomed, USA) for the structure and morphology of cells. Microscopic images obtained by a CCD camera and Digipro software. The cells also were counted in different regions of hippocampus (CA1, CA2, CA3 and DG) by the grade of light microscope. The results are represented a cell count per mm2 tissue. Also some of slides were investigated by Immunohistochemical methods. In these processes, the primary and secondary antibodies were used and Caspase 3 (an

apoptotic cytoplasmic protein) was detected as brown color after making complex with DAB (Diamino Banzydil).

Statistical Analysis:

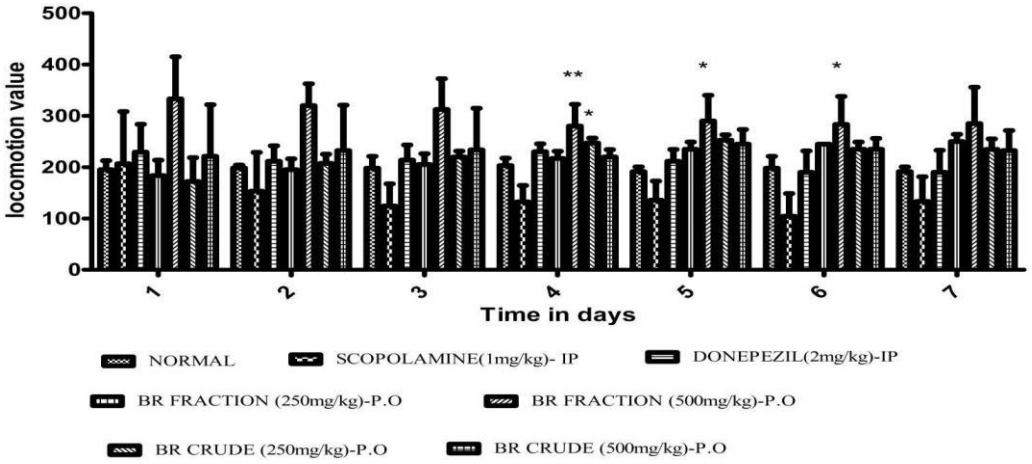
The results were expressed as mean ± SEM (n=6). Statistical analysis was performed using one way ANOVA followed by dunnetts comparison test T. P-values (expressed) calculated against control group and $p<0.001$ were considered.

Results And Discussion:

Locomotor Activity Using Actophoto meter:

Groups	Time in sec						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
I(Normal)	195±17.67	198.33±5.67	198±22.98	203.33±14.28	191.33±9.22	198±22.98	191.33±9.22
II(Control)	206.66±101.72	153±75.93	124.33±43.16	132.33±32.06	135.66±37.25	104.33±44.36	133.33±48
III(Standard)	229±54.71	211.66±30.06	213.66±29.71	230±15.41	211.66±23	190±41.68	190±43.01
IV(Fraction Low dose)	183.5±30.40	195±21.21	205±21.21	217±14.14	235±14.14	245±0	250±14.14
V(Fraction High dose)	333±82.02	320±42.42	312.5±60.10	280±42.42	290±50.20	283.5±54.44	285±70.71
VI(CrudeLow dose)	172±46.66	207.5±17.67	220±11.31	247±9.89	252.5±10.60	235±14.14	234.5±20.50
VII(Crude High dose)	221±101	232.5±88.38	233.5±81.31	220±14.14	245±28.28	235±21.21	232.5±38.89

Table 3: locomotor activity using actophotometer



Graph 2: locomotion activity

Data represents as Mean±SEM (n=6), one way ANOVA followed by Dunnetts multiple comparison test was applied to compare the differences within the groups.* compared with diseased group*P<0.01, **P<0.001.BR-Biophytum reinwardtii plant extract.

Active Response By Jumping Box:

Groups	Time in sec						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
I (Normal)	4.66±1.08	2.5±0.93	1.5±0.35	1±0	1.16±0.20	1±0	1.16±0.204
II (Control)	5±0.70	5.96±1.37	5.56±0.69	5.33±0.40	4.83±0.20	1.5±0.35	1.5±0.35
III (Standard)	6.6±0.81	4±0.70	3.16±0.20	1.33±0.20	1.83±0.40	1.5±0.35	1.5±0.35
IV (FractionLow dose)	2.5±0.70	1.25±0.35	1.25±0.35	1.2±0.28	1.2±0.28	1.1±0.14	1±0
V (FractionHigh dose)	2±0	2.5±0.70	2.33±1.63	1.5±0.70	1.5±0.70	1±0	2.4±1.59
VI (CrudeLow dose)	2±0	2±0	1.65±0.21	1.25±0.35	1.25±0.35	1.1±0.14	1±0
VII (CrudeHigh dose)	2.5±0.70	2.25±1.06	1.4±0.14	1.75±0.35	1.25±0.35	1±0	1±0

Table 4: Response activity by jumping box

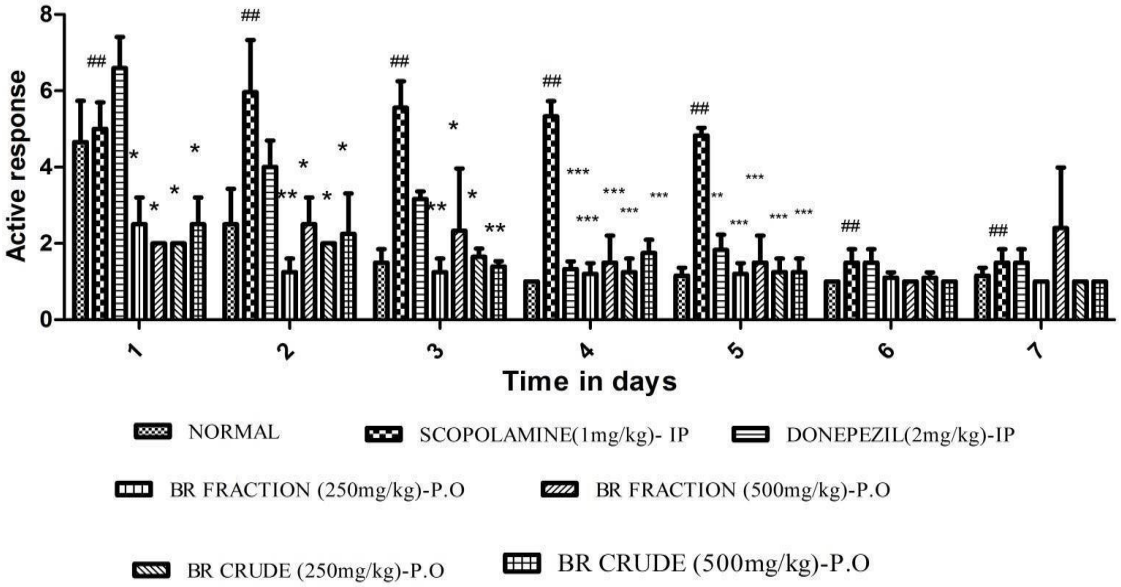
Graph 3: Active response by jumping box

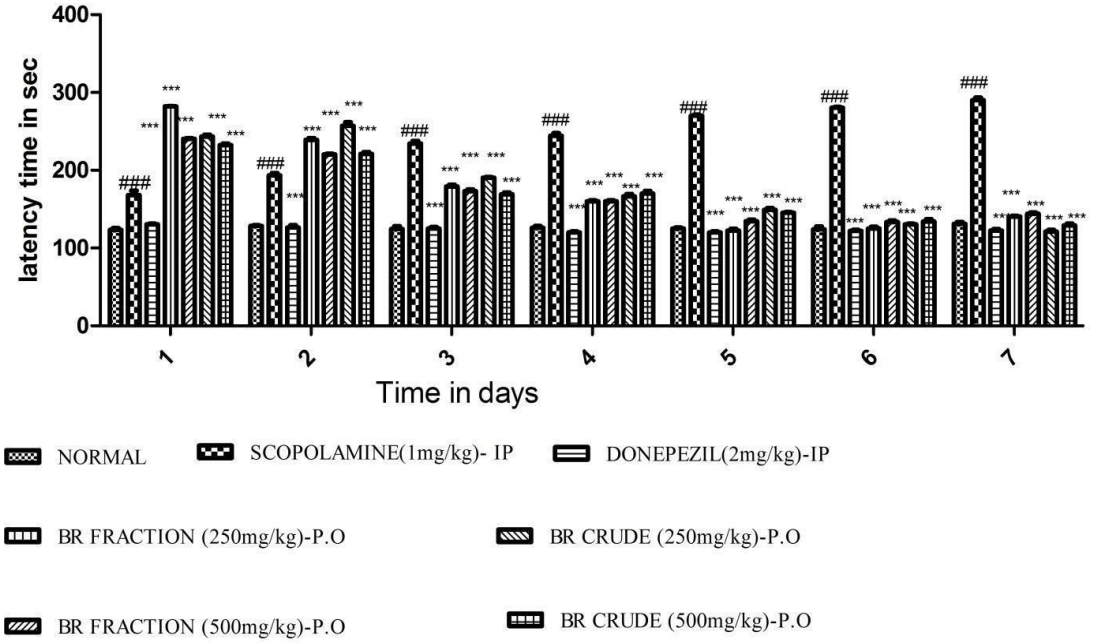
Data represents as Mean±SEM (n=6), one way ANOVA followed by Dunnetts multiple comparison test was applied to compare the differences with in the groups.* Compared with diseased group*P<0.01,**P<0.001. ***P<0.0001,#compared with normal group #P<0.01,##P<0.001,BR-Biophytum reinwardtii plantextract.

Active avoidance by Elevated plus maze:

GROUPS	Time in sec						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
I	123.66±2.27	128.66±0.81	124.66±3.55	126.33±2.160	125±1.41	124±3.74	131±2.54
II	168.33±5.40	193.66±2.85	234.66±3.18	244.66±3.18	270±1.41	280.66±0.81	290±3.53
III	130.66±0.81	126.66±1.08	125.33±1.77	120±1.41	120±1.41	122±1.41	122.61±1.77
IV	282±1.41	239.33±2.16	179.33±2.16	160±1.41	122.66±2.16	125.33±2.16	140.66±0.81
V	240.66±0.81	220.66±0.81	173.33±2.16	160±1.41	134.66±1.77	133.66±1.77	144.33±1.47
VI	243.66±2.27	257.33±4.54	190.66±0.81	166.66±2.94	149.33±2.16	130.66±0.81	121±2.54
VII	232.66±1.77	221.33±2.27	169±2.54	170±3.53	145.66±0.81	134.33±2.85	129±2.54

Table 5: Active avoidance by Elevated plus maze





Graph 4: Active avoidance by Elevated plus maze

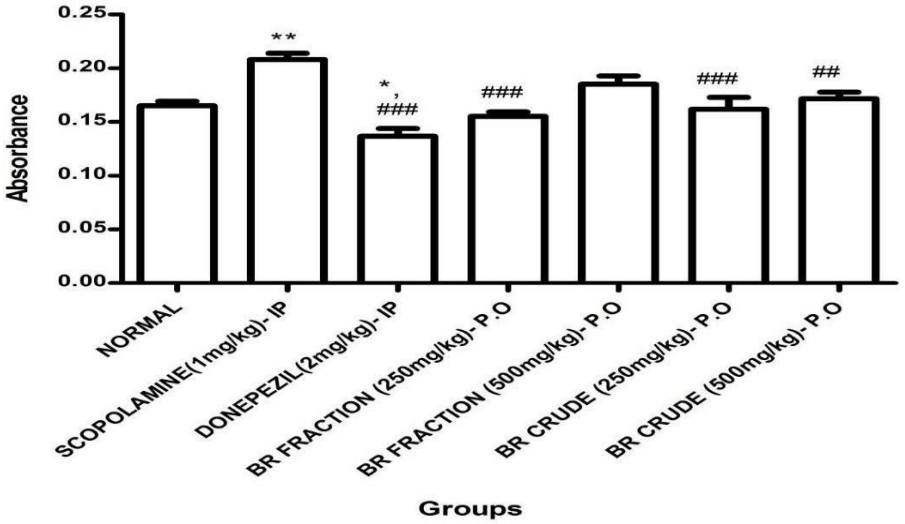
Data represents as Mean±SEM (n=6), one way ANOVA followed by Dunnetts multiple comparison test was applied to compare the differences within the groups.* compared with diseased group*P<0.01, **P<0.001. ***P<0.0001,#compared with normal group,###P<0.0001.BR-Biophytum reinwardtii plant extract.

Acetyl Cholinesterase: (u/mg/protein)

Groups	R_1	R_2	R_3	R_4	R_5	R_6	*Mean±SEM*
I (Normal)	0.16	0.15	0.16	0.17	0.17	0.18	0.16±0.0046
II(Control)	0.19	0.21	0.20	0.22	0.22	0.81	0.30±0.11
III(Standard)	0.13	0.11	0.13	0.14	0.15	0.16	0.13±0.18
IV(Fraction lowdose)	0.14	0.15	0.16	0.17	0.16	0.15	0.15±0.16
V(Fraction highdose)	0.17	0.16	0.18	0.20	0.19	0.21	0.18±0.17
VI(crude lowdose)	0.12	0.19	0.15	0.15	0.17	0.19	0.16±0.012
VII(Crude highdose)	0.19	0.18	0.16	0.15	0.18	0.17	0.17±0.0006

Table 6: Acetyl cholinesterase levels

Effect of MEBR on Acetyl cholinesterase:



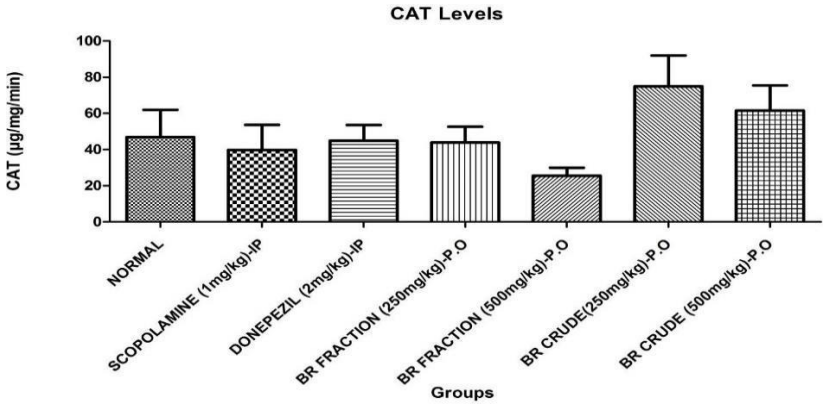
Graph 5: Effect of MEBR on Acetyl cholinesterase

Data represents as Mean±SEM (n=6), one way ANOVA followed by Dunnetts multiple comparison test was applied to compare the differences within the groups.* compared with diseased group*P<0.01, **P<0.001. #compared with normal group, ###P<0.0001. BR-Biophytum reinwardtii plant extract.

Estimation Of Catalase Levels:

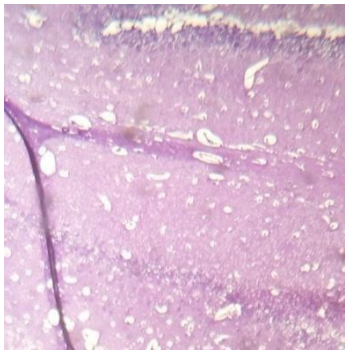
Treatment groups	CAT(µM/mg/min) Brain Homogenate sample
Normal	46.84±15.08
Disease control	39.76±13.90
Standard	44.85±8.65
Fraction low dose (250mg /kg)	43.89±8.71
Fraction high dose(500mg /kg)	25.55±4.42
Crude low dose (250mg/kg)	74.96±17.03
Crude high dose(500mg/kg)	61.58±13.90

Table 7: catalase levels in different groups

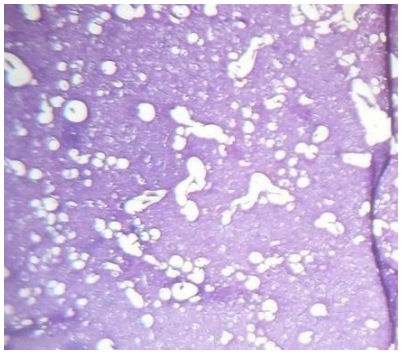


Graph 6: Comparison of Catalase levels

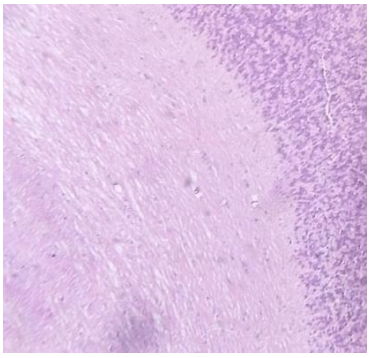
HISTOPATHOLOGICAL STUDIES:



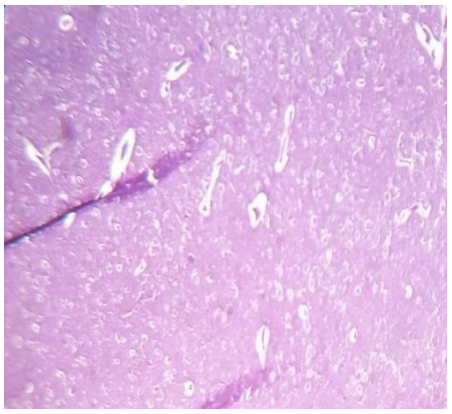
NORMAL (A)



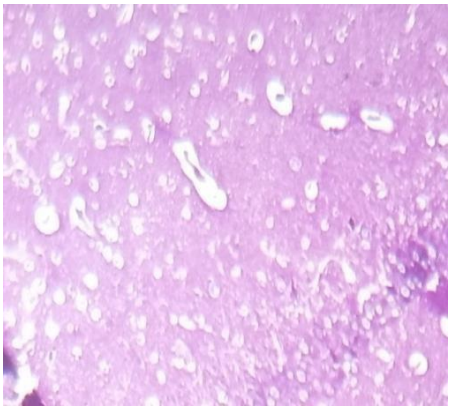
CONTROL (B)



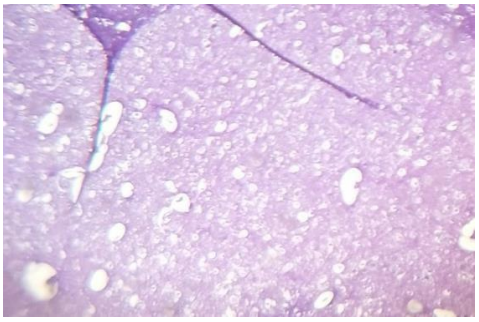
STANDARD (C)



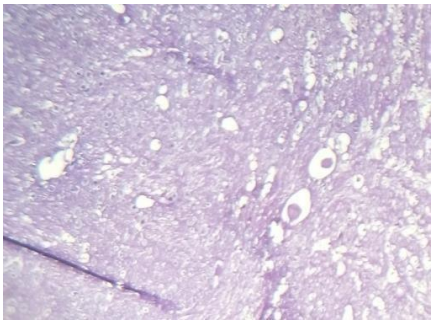
FRACTION LOW DOSE (D)



FRACTION HIGH DOSE (E)



CRUDE LOW DOSE (F)



CRUDE HIGH DOSE (G)

(A) Normal control rat showing normal histological structure of Hippocampus

(B) Scopolamine induced dementia rat showing severe congestion of blood

capillaries with perivascular edema (Scars) together with edema and amyloid plaques in hippocampus.

(C) Scopolamine induce dementia rat treated with donepezil showing diffuse gliosis and shrinkage of nuclei in pyramidal cell in hippocampus.

(D) When compared with control, fraction treated rat show less formation of amyloid plaques in hippocampus region of brain

(E) When compared with control, fraction high dose treated rat show less formation of amyloid plaques in hippocampus region of brain

(F) When compared with control, crude low dose treated rat show less formation of amyloid plaques in hippocampus region of brain

(G) When compared with control, crude high dose treated rat show less formation of amyloid plaques in hippocampus region of brain

When comparing fraction and crude extract treated groups fraction treated group shows better result.

Conclusion:

MEBR fractions (250 and 500 mg/kg) significantly attenuate scopolamine-induced dementia by improving the learning, memory, antioxidant potentiality and anti-acetylcholinesterase activity. Therefore, this extract can be a potential novel therapeutic strategy for controlling neurodegenerative dementia especially AD. Yet, advance studies are needed to expose the possible mechanism of action.

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