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Research Article

Neuroprotective evaluation of *Marsilea quadrifolia* L. and *Salvinia molesta* D.S Mitchel in aluminium chloride induced Alzheimer disease

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Abstract

Phenolic compounds are very effective for the cure of various neurological and pathological diseases. Current study was conducted to estimate the Phenolic compounds through HPLC and to perform the anti-Alzheimer activity from two aquatic Leptosporangiate ferns; *Marsilea quadrifolia* L. and *Salvinia molesta* D. Crushed leaves were subjected to Microwave Assisted Extraction to prepare plant leave ethanolic extract of plants under study. Aluminum chloride induced Alzheimer's disease model in rats by estimating behavioural and biochemical parameters were measured for ethanolic extract of both plants. Different behavioural tests were performed for neuromuscular coordination estimation and hole board test for exploratory behaviour were performed. The levels of AChE inhibitory activity and antioxidant enzymes were estimated on brain homogenates and Histopathological analysis were also performed. Phenolic compounds of leaf extracts were noted for HPLC chromatograms of standard compounds with Gallic acid (33.76 ± 0.08) higher in *M. quadrifolia* L. and Salicylic acid (12.45 ± 0.07) higher in *S. molesta* D. S Mitchel. Histopathological analysis showed that *M. quadrifolia* and *S. molesta* reversed the neurofibrillary tangles, degenerative changes, neuronal loss and neuroinflammation in the brain architecture. Both ferns at a dose of 500 mg/kg and 1000mg/kg significantly improved the Aluminium chloride induced neurotoxicity by modulating cognitive disabilities and motor dysfunctions, and normalized the biochemical alteration of acetylcholinesterase and antioxidant enzymes. In conclusion, both plants *M. quadrifolia* and *S. molesta* improved behavioural and biochemical

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parameters and therefore will be effective in treatment of neurodegenerative disorders via countless underlying mechanistic pathways.

Keywords: anti-Alzheimer activity; *Marsilea quadrifolia*; neurodegenerative disorders; neurofibrillary tangles; neuroinflammation; *Salvinia molesta*

Introduction

Indigenous knowledge of medicinal pteridophytes can be utilized to elucidate the therapeutic use of plants by conducting ethnobotanical studies in present and past culture all over the world. To authenticate the medicinal activities of plants and their efficacy in chronic illness quantitative ethnobotanical SWOT analysis is used.

In order to decrease loss to environment by pesticides the biological systems have established a defensive means which contain antioxidant molecules. When toxic agents use the endogenic levels of antioxidants, exogenous antioxidative and protective compounds may be replaced to reduce adversarial health outcomes (Kakoolaki *et al.* 2013). The examination of new antioxidants as therapeutic agent is an active type of biochemistry. A large number of organic types of antioxidant molecules have been studied as preventive and Natural therapeutic agents. Propolis is a form of a phenolic compound which may be capable of stopping apoptosis by declining oxidative stress. (Sevindik *et al.*, 2020). In atherosclerotic risk factors such as hypertension Reactive oxygen species (ROS) production has increased. This is important because of essentially every aspect of atherosclerotic lesion formation has been affected by oxidative events. (Zeliha *et al.* 2013). superoxide dismutase make converts seperoxide anions radicals O_2. To H_2O_2 when ROS are increased. Catalase (CAT) removes H_2O_2 by breaking down directly to O_2. Thus, H_2O_2 is reduced, suggesting that an imbalance between oxidant and antioxidant mechanisms is a contributing factor (Daglıa *et al*. 2014).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by behavioural and personality impairments along with dementia. AD is a condition of high β-amyloid level and neurofibrillary tangles due to mutation or duplication of β-amyloid precursor proteins (APP) and a presenilin-encoding gene for proteolytic enzymes (Tanzi and Bertram, 2005). Genetic, environmental factors and aging are mainly inculpated in pathogenesis of neurodegenerative disorder. AD is slowly progressive disorder and takes years to manifest the deceptive clinical symptoms which eventually lead to degenerated cognition (Urdinguio *et al.* 2009). AD develops with abnormal functioning of brain, initially by failure to create new memories as it is difficult to associate new information and leads to rapid forgetting (Weintraub *et al.*, 2012)Risk of cognitive (mental) aging and AD increased due to neuro-inflammation induced by oxidative stress and mitochondrial dysfunctions (Irwin and Vitiello, 2019).

In normal physiological state, the production of free radicals and oxidative stress are controlled by antioxidant enzymes, but in AD, these enzymes fail to perform their regular function in the brain. Development of pathology in AD starts from the perirhinal region to the hippocampus complex and eventually to temporal lobes with the basal forebrain (Bredesen, 2009). AD places the socioeconomic burden on patient and on caregivers through impairment of motor functions and mentation resulting in poor quality of life. AD linked behavioural and neuromuscular coordination impairment markedly changes the lifestyle of patient Education, gender, aging, apolipoprotein E ε4 allele and genetic mutations are significant risk factors for AD. Diabetes, cardiovascular disease and smoking also increase the risk of dementia and AD (Zucchella *et al*., 2015).

Acknowledging the medicinal activities of these ferns, the current work is designed to elucidate the neuroprotective potential of *Marsilea quadrifolia* L. and *Salvinia molesta* D. S Mitchel through modulation of behavioural changes, locomotor functions, antioxidant enzymes and neuro-inflammatory biomarkers.

Materials and Methods

Collection and authentication

Two Pteridophytes *M. quadrifolia* L. and *S. molesta* D. S Mitchel collected from two different sites i.e., Lahore and Mandi Bahauddin Pakistan. Plants were identified by the taxonomists of Department of Botany GCU Lahore, a voucher sample was submitted in herbarium and provided with voucher numbers. GC. HERB. BOT. 3092 Voucher number of *M. quadrifolia* L. and GC. HERB. BOT. 3091 Voucher number of *S. molesta* D. S Mitchel.The plants were collected along with their Rhizome, and after rinsing were subjected to drying process. Plants were kept at room temperature overnight for 12 hours and then were kept in Hot air oven for 24 hours at 45 °C. Then leaves of plant were crushed into fine powder with help of pestle and mortar, and Grinder.

Microwave Assisted Extraction (MAE)

Dried leaf powder of each plant was subjected to (MAE) for extraction as reported in our previous work. Briefly, 300 g plant powder into 500 mL of ethanol was heated in microwave for 3 cycles (900 W) at 70 °C (each cycle of 6 minutes with 1 minute pause between each cycle). The ethanol elution was subjected to rotary evaporation at 45 °C to get semisolid extract (Lateh *at al.,* 2019).

High Performance Liquid Chromatography (HPLC) Analyses

Plant extracts were dissolved in methanol and filtered through 0.2 µm membrane filter (Millex-HV) before injecting into HPLC. The HPLC system (Shimadzu LC-20AT) equipped with auto-sampler (SIL-20A), column oven (CTO-20A), and diode array detector (SPD-M20A). Analytical column- Purospher Star RP-18 end capped 5 µm 100 Å (250 × 4.60 mm, Merck, Germany) coupled with a guard column (KJO-4282, Phenomenex) was used. Mobile phase composed of (A) 0.1% acetic acid and (B) methanol and the composition gradient was: 25% (B) for 10 min; 40% (B) for 15 min; then 50%, 60%, 80% and 100% (B) for every 10 min (Jamshed, Siddiqi *et al.,* 2019). Flow rate was 0.8 mL min^{-1}. Phenolic compounds were identified by comparing retention time and UV-Vis spectra of chromatographic peaks with that of authentic reference standards at 280 nm (Grad and Chengelis, 1988).

Animals

Healthy female Wistar rats were acclimatized for 1 week prior to experimental work in animal house of Government College University, Faisalabad. Standard laboratory conditions were provided with free access to water and food. Animal study was conducted after getting approval from institutional review board, Government College University, Lahore. According to OECD 425 guidelines for oral acute toxicity study nulliparous and non-pregnant female animals were selected. To investigate the neuroprotective effect of plants, extract male rats were selected.

Acute oral toxicity study

Limit test dose 2000 mg/kg for each plant extract was provided to single female rat and four more animals were treated with same dose. Animals were kept under observation after initial dose to final dose to note behavioural change and mortality. After 14 days body weight was noted and animals were sacrificed under anaesthesia to collect blood samples and vital organs for biochemical analysis and histopathological analysis (Naz *et al.*, 2022).

Experimental induction of Alzheimer's disease

Alzheimer's disease like symptoms was induced by administration of aluminium chloride 150 mg/kg for 28 days. Aluminium intoxication encourages the oxidative stress through exacerbation of reactive oxygen species activities and via production of proximities which markedly disrupt neuronal function. Aluminium chloride by interfering the TCA cycle induced hypometabolism of glucose and amyloidogenic amyloid precursor protein production which perpetuated the oxidative insult (Worasuttayangkurn *et al.*, 2012).

Experimental study design

Healthy male rats were taken and divided into six groups.

Group 1 was given with 1 ml/kg distilled water.

Group 2 was treated with 100 mg/kg aluminum chloride.

Group 3 was treated with 100 mg/kg aluminum chloride and rivastigmine.

Group 4 was treated with 100 mg/kg aluminum chloride and deal with *M. quadrifolia* and *S. molesta.*

Group 5 contained 100 mg/kg aluminum chloride and 500 mg/kg *M. quadrifolia* and *S. molesta.*

Group 6 contained 100 mg/kg aluminum chloride and 1000 mg/kg *M. quadrifolia* and *S. molesta.*

Body weight and behavior were recorded before and after the experiment.

Behavioural studies

Morris water maze task

Circular plastic tub filled with water. A platform was set at centre of pool for training trails. In start animals escaped through submerged platform in 20 seconds and after some day's platform was made invisible by mixing Milk powder and animals taken 120 seconds (Morris 1984).

Open field test

Floor of plywood chamber divided in equal squares & red lines drawn in central squares. Disinfected chamber with alcohol. Total distance & lines noted (Morris 1984). Grooming, defecation, time spent in central area and rearing recorded.

Passive avoidance test

Plexiglas chamber with grillwork at floor connected with battery. Rostrum of wood was set at centre. Time of heaped up and step-down latency observed. To avoid shock, animals with AD pathology oppose their intrinsic biasness (Ataie *et al.*, 2010).

Y-maze test

Wooden apparatus with three arms attached. Rats were placed at start of arm and recorded number of entries and triads to determined spontaneous alternations. In this test animals spontaneously alter arms of maze (Sohanaki *et al.*, 2016).

$$Spontaneous\ Alteration\ \% = \frac{Total\ no.of\ trials}{Total\ no.of\ arm\ enteries - 2} \times 100$$

Laterality index was calculated to investigate the side preference of animal by following equation:

$$Laterality\ index = \frac{Movement\ toward\ left\ arm - Movement\ toward\ right\ arm}{Movement\ toward\ left\ arm + Movement\ toward\ right\ arm}$$

Hole board test

Plexiglas apparatus with 16 equally divided holes in floor was constructed. Rats leave on ground. Distance in periphery and centre, number of head dipping in holes noted. It used for estimation of neophilla, stress and anxiety in AD rats (Aydin *et al.,* 2016).

Wire hanging test

Horizontal stainless-steel grids placed on wooden walls. Animals were gently hanged on grid downward in upright direction. Hanging time was recorded. This test was used to detect neuromuscular strength and endurance of animal (Borght *et al*., 2007).

Elevated plus-maze task

Two open and closed arms apparatus constructed with central wooden platform. Time was noted to move from open arm to closed arm. To analyse cognition, memory deficit and learning the transfer latency was recorded (Tillerson and Miller, 2003). This task was performed to determine anxiety and spontaneous behaviour patterns in AD rodent.

Estimation of biochemical parameters

Tissue homogenate preparation

Each animal was feasibly killed by giving anaesthetic isoflurane and the brain was taken off by euthanasia. Brains were washed with frozen NS (normal saline) and cooled at -80 °C in a biomedical freezer. In a tissue homogenizer the brain tissues were homogenized; supernatant was taken by centrifuging at 800 rpm for 30 minutes at 4 °C (Saleem *et al.*, 2019).

Estimation of Melondialdehyde (MDA level)

Brain homogenate mixed with Sodium dodecyl sulphate, acetic acid, triobarbituric acid and poured in distilled water. Mixture heated in water bath and cooled. Centrifugate at 4000 rpm and standard curve plotted of different concentration of thiobarbituric acid (Parle *et al.*, 2005).

$$\mathrm{Y} = 0.0278\mathrm{x} - 0.2485$$

Estimation of catalase (CAT) activity

Brain homogenate was mixed with phosphate buffer and H_2O_2. Optical density was recorded at 240nm (Komada *et al.*, 2008).

$$CAT\ activity = \frac{\delta O.D.}{E\ \times Vol.of\ sample\ (ml) \times mg\ of\ protein}$$

Here E = 0.071 mmol cm^{-1} (the extinction coefficient of hydrogen peroxide) and δO.D. = absorbance shift per minute.

Estimation of superoxide dismutase (SOD) activity

Mixture was prepared by mixing sodium phosphate buffer, tissue homogenate, phenazine methosulphate, nitro blue tetrazolium and incubated at 30 °C. After addition butanol, allowing to settle down and centrifuged at 1000 rpm. Intensity of chromogen recorded at 560 nm (Lakshmi *et al.*, 2015).

Estimation of reduced glutathione (GSH) level

Mixture was composed by mixing potassium chloride, tissue homogenate, cold distilled water and trichloracetic acid. Centrifuged at 3000 rpm for half hour. At 412 nm, absorbance of sample and blank was noted (Botsoglou *et al.,* 1994).

$$Concentration\ of\ GSH = \frac{Absorbance}{Extinction\ coefficient} \times Dilution\ factor$$

Here A is the absorbance, D is dilution factor and E is molar extinction coefficient (C=13,000 M^{-1} cm^{-1}).

Estimation of acetylcholinesterase activity

Small quantity of tissue homogenate was mixed with phosphate buffer, dithiobis nitrobenzoic acid, acetylcholine iodide. Yellow color appeared and absorbance recorded at 412 nm (Aebi 1984).

$R = 5.74\times10-4 \times A/CO$

PCR amplification in real-time

To determine the genes expressions that are associated with Alzheimer's disease PCR technique was performed by using the following primers α-synuclein, IL-1α, IL-1β, TNF-α, AChE (acetylcholinesterase), β-secretase, and ABPP (β-amyloid precursor protein). RNA extracted using TRIzol method was quantified by measuring 260/280 nm absorbance ratio using nanodrop spectrophotometer. RNA was then transcribed into cDNA using Thermo Scientific cDNA kit. The level of gene expression was assessed via qRT-PCR by using GADPH as a house-keeping gene. For the experiment, a forward primer (0.5 µL) was used along with corresponding reverse primer (0.5 µL), and cDNA volume of 5 µL plus cyber green volume of 5 µL was placed in microplate wells. Thermal cycler was programmed at forty cycles of denaturation at 95 °C, annealing at 60 °C and extension at 72 °C by putting microplate in it (Saleem and Kannan, 2018). Gel electrophoresis was performed by pipette together 5 µL sample with 5 µL DNA ladder to visualize bands pattern.

Statistical analysis

The result was demonstrated using a graph pad. Analysis of variance (ANOVA) was applied to data to predict the significance difference between variables.

Results

Following results of pharmacological activities of *M. quadrifolia* and *S. molesta* were obtained, HPLC chromatograms of standard compounds and plant leaf extracts of *M. quadrifolia* Indicated that Gallic acid 33.76 ± 0.08 and Hydroxybenzoic acid 31.69 ± 0.01 showed the highest values, Table 1 given below shows the values of HPLC for *M. quadrifolia.* HPLC chromatograms of standard compounds and leaf extracts of *S. molesta* showed the Coumaric acid 9.73 ± 0.05 and Salicylic acid 12.45 ± 0.07 were found to be highest. Table given below shows the values of HPLC for *S. molesta.* (Table 1; Figure 1).

Table 1. Phenolic composition (mg g^{-1} dry weight) of leaf extracts of *M. quadrifolia and S. molesta*

Compounds	*Salvinia molesta*	*Marsilea quadrifolia*
Pyrogallol	Nd	0.62 ± 0.01
Gallic acid	5.50 ± 0.02	33.76 ± 0.08
Catechol	3.19 ± 0.01	1.49 ± 0.01
Hydroxybenzoic acid	6.28 ± 0.03	31.69 ± 0.01
Chlorogenic acid	Nd	0.19 ± 0.01
Coumaric acid	9.73 ± 0.05	Nd
Ferulic acid	Nd	Nd
Rutin	Nd	Nd
Salicylic acid	12.45 ± 0.07	Nd
Cinnamic acid	0.13 ± 0.00	0.15 ± 0.00
Quercetin	0.72 ± 0.01	0.59 ± 0.01

Nd, not determined

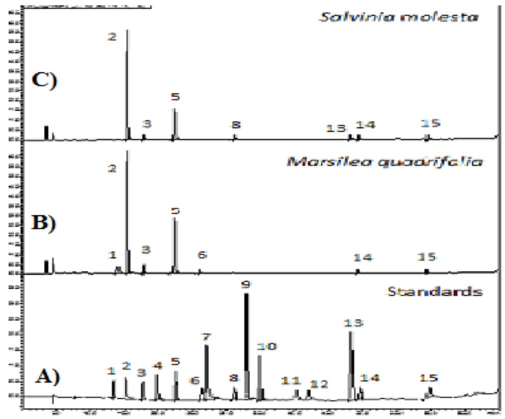

Figure 1. HPLC chromatograms of standard compounds and leaf extracts of *Marsilea quadrifolia* and *Salvinia molesta*. Individual peaks showed phenolic compounds i.e., Pyrogallol (1), Gallic acid (2), Catechol (3), Catechin (4), Hydroxybenzoic acid (5), Chlorogenic acid (6), Syringic acid (7), Coumaric acid (8), Ferulic acid (9), Rutin (10), Sinapic acid (11), Coumarin (12), Salicylic acid (13), Cinnamic acid (14), and Quercetin (15)

Behavioural studies

<u>Passive avoidance task</u>

The test was performed to examine to estimate learning and memory in rats by giving foot shock in one of the chamber compartments. It was noted that learning and memory activity was significantly ($P < 0.05$) decreased in DC group in comparison with C group. SD, Mq5, Sm5 and Mq10, Sm10 increased the learning and memory investigation of rats significantly ($P < 0.05$) then the DC group. Mq10 and Sm10 showed significantly higher learning and memory examination as compared with Mq5 and Sm5 groups (Figure 2a).

<u>Hole board test</u>

It was investigated that head dip count decreased significantly ($P < 0.05$) in DC, Mq10, Sm10 groups in comparison to the C, SD, Mq5 and Sm5 groups. Locomotory and exploratory behaviour were impaired by haloperidol in DC group (Figure 2b).

<u>Wire hanging test</u>

Wire hanging test was used to discover the potential or fall-off time which condensed significantly ($P<0.05$) in the DC group in comparison to the C group. Fall-off time was significantly ($P<0.05$) improved in dose dependent manner in Mq5, Sm5 and SD groups when compared to the DC group. Also, Mq10, Sm10 groups showed strengthened neuromuscular coordination by improving the hang time compared toMq5, Sm5 groups (Figure 2c).

<u>Elevated plus maze</u>

This test was performed to assess anxiety-related behaviour. Their behaviour was recorded by means of a video camera mounted above the maze and analysed using a video tracking system. The preference for being in open arms over closed arms was noted. It was noticed that anxiety-related activity was significantly ($P < 0.05$) decreased in DC group in comparison with C group. SD, Mq5, Sm5 and Mq10, Sm10 significantly ($P < 0.05$) increased the anxiety-related investigation of rats compared to DC group. In contrast, Mq10 and Sm10 groups showed significantly higher anxiety-related examination compared to Mq5, Sm5 groups (Figure 2d).

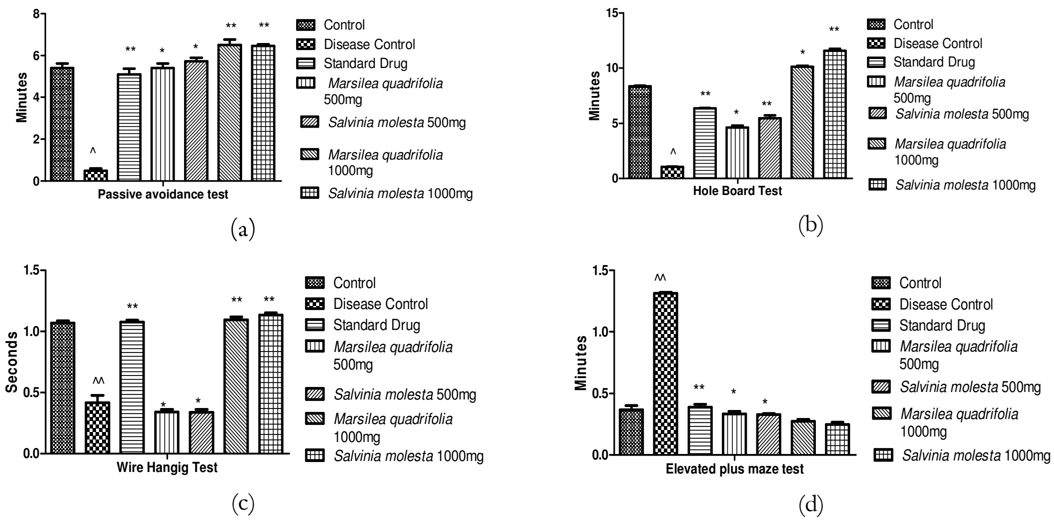

Figure 2. Behavioural changes in rats during Alzheimer activity, (a) Passive Avoidance test, (b) Hole Board test, (c) Wire Hanging test (d) Elevated Plus Maze test. Values are expressed as mean ± SEM, (n = 6), ***P < 0.05 and ns (non – significant) as compare to control

Open-field test

The test was performed to examine locomotion and anxiety such as frequency of raising and number of lines crossed. It was determined that locomotor activity was expressively (P<0.05) declined in DC group in comparison with C group. SD, Mq5, Sm5 and Mq10, Sm10 showed significantly (P < 0.05) enhanced the movement and anxiety in rats compared to DC group. Binary and ternary insertion centres of the dose of Mq10 and Sm10 showed significantly higher locomotive activity and anxiety as compared to Mq5 and Sm5 groups (Table 2).

Y Maze Test

Y Maze test was used for measuring the cognitive deficits and willingness of rodents to explore new environments. The number of arm entries and the number of triads were recorded in order to calculate the percentage of alternation. It was noted that cognitive deficits activity was expressively (P<0.05) decreased in DC group compared to C group. SD, Mq5, Sm5 and Mq10, Sm10 groups significantly (P < 0.05) increased the cognitive deficits investigation in rats compared to DC group (Table 3).

Morris water maze test

Morris water maze test was used for measuring spatial learning and memory of rats. Rats were placed in a large circular pool of water and observed to escape from water onto a hidden platform which was normally identified by using spatial memory only. It was noted that spatial learning and memory was expressively (P<0.05) decreased in DC group compared to C group. SD, Mq5, Sm5 and Mq10, Sm10 groups significantly (P < 0.05) increased the spatial learning and memory activity of rats compared to DC group (Table 4). Eyes, salivation, respiration, urination colour, faces consistency mucus membrane, sleep and gait were found to be normal in both plants during behavioural studies, Elevation of fur in first 2 hours after dose were seen due to handling stress and somatomotor activity were slight increase in both plants. There were not seen itching, convulsion and tremor, coma and mortality in any group of both plants (Table 5 and 6).

Table 2. Effect of *M. quadrifolia* and *S. molesta* on open field test in $AlCl_3$ induced AD model

Groups	Total no. of lines (Crossed)	Freezing (Seconds)	Rearing/ 10 min	Grooming/ 10 min
Control	27.00±0.5	0.00±0.00	6.00±0.2	3.00±0.5
Disease control	10.00±0.00 ***	82.0±0.5 ***	0.00±0.00 ***	0.00±0.00 ***
Standard drug	24.66±0.6 ns	11.33±0.9 **	4.00±1.5 ns	3.98±0.8 ns
M. quadrifolia 500 mg (Mq5)	18.00±0.5 ns	17.00±0.5 **	2.78±1.2 ns	1.56±0.3 ns
S. molesta 500 mg (Sm5)	17.50±0.5 **	16.00±0.5 **	3.68±0.3 ns	1.78±0.3 ns
M. quadrifolia 1000 mg (Mq10)	12.00±0.5 ns	0.23±0.00 ns	2.50±0.5 ns	2.00±0.5 ns
S. molesta 1000 mg (Sm10)	11.05±0.5 ns	0.22±0.00 ns	2.75±0.5 ns	1.98±0.5 ns

Values are expressed as mean ± SEM, (n = 6), *** (P < 0.001), ** (P < 0.01) and ns (non – significant) as compare to control.

Table 3. Effect of *M. quadrifolia* and *S. molesta* on Y- maze test in AlCl 3 induced AD model

Groups	Total no. of Arm entries	Total no. of Triads	% Spontaneous Alteration	Laterality Index
Control	12.00±0.6	3.02±0.00	42.00±0.5	0.12
Disease control	2.35±0.33 ***	0.00±0.00 ***	0.00±0.0 ***	-0.33
Standard drug	10.00±0.5 ns	3.38±0.3 ns	35.00±0.5 ns	0.14
M. quadrifolia 500 mg (Mq5)	5.68±0.33 *	2.02±0.00 *	30.14±0.4 **	0
S. molesta 500 mg (Sm5)	6.00±0.33 *	2.75±0.00 ns	28.14±0.41 **	0
M. quadrifolia 1000 mg (Mq 10)	8.00±1.7 ns	2.05±0.00 ns	20.00±0.49 ns	0.1
S. molesta 1000 mg (Sm10)	6.89±1.7 ns	2.25±0.00 ns	22.0±0.47 ns	0.1

Values are expressed as mean ± SEM, (n = 6), *** (P < 0.001), ** (P< 0.01) and ns (non – significant) as compare to control.

Table 4. Effect of *M. quadrifolia* and *S. molesta* on Morris water maze in $AlCl_3$ induced AD model

Groups	Quadrat 1	Quadrat 2	Quadrat 3	Quadrat 4
	Escape Latency	**In Seconds**		
Control	20.23±1.2	25.67±1.3	20.0±1.6	18.3±1.2
Disease control	45.33± 1.2 ***	41.00±2.2 ***	41.66±1.5 ***	44.33±1.7 ***
Standard drug	24.00±0.6 ns	25.33±0.8 ns	21.00±0.5 ns	17.00±0.5 ns
M. quadrifolia 500 mg (Mq5)	30.00±1.2 **	25.33±0.3 **	27.00±0.7 *8	29±0.5 **
S. molesta 500 mg (Sm5)	28.00±1.6 *	22.33±0.6 ns	25.00±0.6 **	27±0.6 **
M. quadrifolia 1000 mg (Mq5)	10.00±0.6 ns	12.5±0.3 ns	12.00±0.3 ns	10±0.5 ns
S. molesta 1000 mg (Sm10)	12.00±0.5 ns	11.5±0.3 ns	11.9±0.3 ns	9±0.5 ns

Values are expressed as mean ± SEM, (n = 6), ***(P < 0.001) and ns (non – significant) as compare to control.

Assessment of biochemical parameters

The level of biochemical oxidative stress markers like CAT, SOD and GSH reduced significantly (P < 0.05). MDA level markedly increased (P< 0.05) in the DC group on the administration of haloperidol. Mq5, Mq10, Sm5, Sm10 and SD groups restored the declined level of CAT, SOD and GSH, and the raised level of MDA on compared to DC, Mq10 and Sm10 groups (Figure 3).

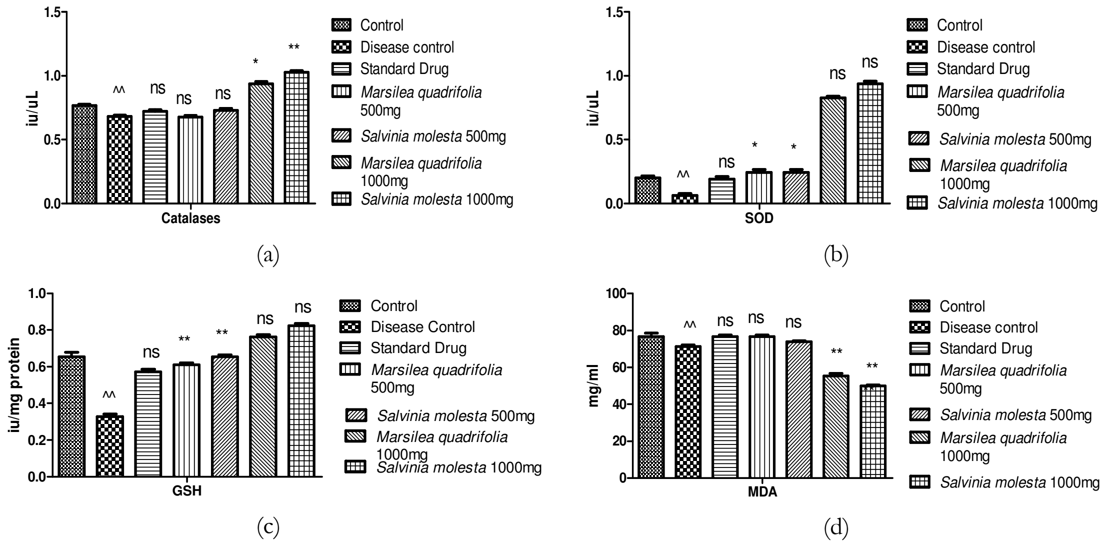

Figure 3. Effect of *M. quadrifolia* and *S. molesta* on antioxidant parameters in $AlCl_3$ induced AD model. A significant increase in levels of (a) catalases, (b) superoxide dismutase; SOD, (c) glutathione; GSH was observed compared to decreased level of, (d) malondialdehyde; MDA in plants treated rat model.

Assessment of acetylcholinesterase

Acetylcholine is the main neurotransmitter associated with cognitive abilities and the basal ganglia regulating motor coordination. The level of acetylcholine was reduced significantly (P< 0.05) in the DC group in contrast to C group. Treatment with *Marsilea quadrifolia* 500 mg, *M. quadrifolia* 1000 mg, *Salvinia molesta* 500 mg and *S. molesta* 1000 mg and SD groups elevated the level of neuromodulators significantly (P < 0.05) on comparison with DC group. *M. quadrifolia* 1000 mg and *S. molesta* 1000 mg groups have more neuroprotective propensity then the Mq5 and Sm5. (Figure 4).

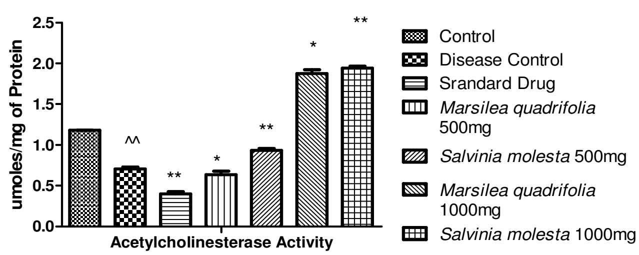

Figure 4. Effect of *M. quadrifolia* and *S. molesta on* acetylcholinesterase activity (µmoles/ mg of proteins) in brain tissue. Values are expressed as mean ± SEM, (n = 6), *** P < 0.05 and ns (non –significant) as compare to control

Examination of histopathological changes in rat brain

Where Control indicated the normal architecture of control group, Neurofibrillary tangles, Lewy body inclusion and depigmentation were observed in the DC group. Each of *Marsilea quadrifolia* and *Salvinia molesta* at 500 mg/kg concentration slightly improved the neurofibrillary tangles and depigmentation. *Marsilea quadrifolia* and *Salvinia molesta* each at 1000 mg/kg concentration markedly improved the neuronal architecture and neuronal loss. Histophatopathological results of brain and vital organs are shown in (Figures 5 and 6)

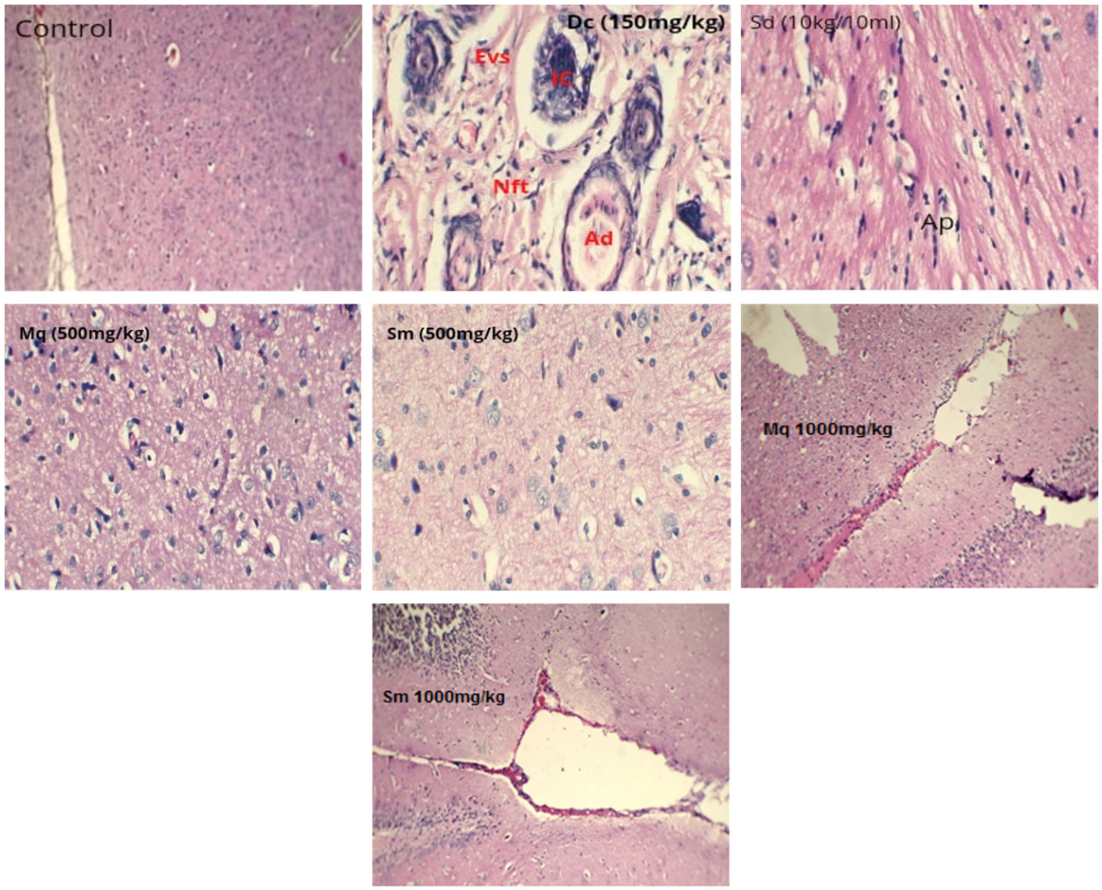

Figure 5. Hematoxylin and eosin staining of transverse brain sections. (Control) indicated normal architecture of control group, Dc: Disease control $AlCl_3$, Sd: Standard drug, Mq (*M. quadrifolia*), Sm (*S. molesta*), IC: Inflammatory cells, Ad: Adema, Evs: Extravesicular spaces, Nft: neurofibrillary tangles, AP: amyloid beta plaques. Indicated neurofibrillary tangles, neurodegeneration, inflammation and extravesicular spaces in disease control group, (Standard). Neurofibrillary tangles and neuronal loss were less in standard treated group. Neurodegeneration was slightly improved in 500 mg/kg and 1000 mg/kg, 500 mg/kg and 1000 mg/kg in *M. quadrifolia* and *S. molesta* treated group and Neuronal loss was improved

Table 5. Behavioural changes during acute toxicity study of *S. molesta*

Behavioural parameters	Observation
Eyes	Normal
Skin and fur	Elevation of fur in first 2 hours after dose due to handling stress
Salivation	Normal
Respiration	Increased
Urination (Colour)	Normal
Faeces consistency	Normal
Somatomotor activity	Slightly increased
Mucus Membrane	Normal
Sleep	Normal
Gait	Normal
Itching	Not found
Convulsion and tremor	Not found
Coma	Not found
Mortality	Not found

Table 6. Behavioural changes during acute toxicity study of *M. quadrifolia*

Behavioural parameters	Observation
Eyes	Normal
Skin and fur	Elevation of fur in first 2 hours after dose due to handling stress
Salivation	Normal
Respiration	Increased
Urination (Colour)	Normal
Faeces consistency	Normal
Somatomotor Activity	Slightly increased
Mucus Membrane	Normal
Sleep	Normal
Gait	Normal
Itching	Not found
Convulsion and tremor	Not found
Coma	Not found
Mortality	Not found

Hematological and biochemical analyses

Tables 7-9 represented the result of blood chemistry, lipid profile and liver and renal function which showed minor alteration in their parameters but within the normal physiological range.

Table 7. Effect of *Marsilea quadrifolia* and *Salvinia molesta* on haematological and biochemical parameters and (Acute oral toxicity)

Parameter	Units	Control Group 10 ml water	*M. quadrifolia* 500 mg/kg	*S. molesta* 500 mg/kg	*M. quadrifolia* 1000 mg/kg	*S. molesta* 1000 mg/kg	*M. quadrifolia* 2000 mg/kg	*S. molesta* 2000 mg/kg
Hb	g/dl	9.66±0.08	12.80±0.26 ns	12.87±0.145 ns	11.03±0.145 ns	10.63±0.20 ns	13.09±0.45 ns	13.63±0.40 ns
TLC	X109/L	14.53±0.17	11.5±0.21*	11.63±0.08*	9.6±0.20*	10.47±0.18*	8.9±0.10*	11.7±0.08*
Total RBC	X1012/L	5.15±0.02	6.46±0.2 ns	6..60±0.42 ns	5.58±0.059 ns	5.39±0.003 ns	6.78±0.049 ns	6.09±0.004 ns
HCT(PVC)	%	33.13±0.08	40.26±0.08 *	40.26±0.08 ns	33.13±0.08 ns	30.70±0.058 ns	36.1±0.09 ns	31.0±0.059 ns
MCV	Fl	45.8±0.10	54.8±0.03 ns	54.8±0.03 ns	47.6±0.12 ns	44.73±0.08 ns	49.6±0.17 ns	48.3±0.09 ns
MCH	Pg	16.35±0.14	19.8±0.05 ns	19.8±0.05*	18.23±0.120 *	18.80±0.058 ns	19.2±0.26*	19.8±0.09 ns
MCHC	%	31.7±0.068	41.03±0.14 ns	41.03±0.145 ns	33.7±0.08 ns	37.6±0.120 ns	35.8±0.098 ns	38.9±0.20 ns
Platelets	X109/L	1044±0.57	714±0.57 **	714±0.57 **	983±0.33 *	1023±0.57 **	1027±0.53 *	1029±0.37 **
D.L. COUNT								
Neutrophils	%	13.66±0.33	17.33±0.33 ns	17.33±0.33 ns	13.66±0.33 ns	17.33±0.33 *	12.67±0.93 ns	18.30±0.32 *
Lymphocytes	%	84.66±0.33	81.33±0.88 ns	81.33±0.88 ns	86.0±0.57 ns	81.3±0.88 ns	88.0±0.67 ns	84.4±0.87 ns
Monocytes	%	1.00±0.003	2.00±0.00 ns	2.00±0.00 ns	1.00±0.00 ns	1.00±0.00 ns	1.00±0.00 ns	1.00±0.00 ns
Eosinophils	%	0.00±0.00	1.00±0.00 ns	1.00±0.00 ns	1.0±0.00 ns	1.00±0.0 ns	1.0±0.00 ns	1.00±0.00 ns

Values are expressed as means ± SEM, (n = 5), *** P< 0.001 and ns (non – significant) in comparison to control groups.

PCR amplification in real-time

Primer blast and Gene bank was used to purpose the following primers; α-synuclein, IL-1β, ABPP, AChE, TNF-α, β-secretase and IL-1α by applying GADPH as an internal reference gene. The gene levels of mRNA expression named alpha-synuclein (α-synuclein), interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α), beta-amyloid precursor protein (ABPP), interleukin 1 alpha (IL-1α), beta secretase (β-secretase) and acetylcholinesterase (AChE) were amplified expressively (P< 0.05) on execution of haloperidol in C group when compared with DC group. Treatment with Mq5, Sm5, Mq10, Sm10 and SD were suggestively downregulated the rise level of genes of mRNA expression on comparison with DC group. Mq10 and Sm10 appreciably reduced the severity of mRNA expressions than Mq5 and Sm5. Results are shown in Figure 7.

Table 8. Effect of *Marsilea quadrifolia* and *Salvinia molesta* on lipid profile (Acute toxicity study) 2000 mg/10 ml

Name of tests	Control Group	*Salvinia molesta*	*Marsilea quadrifolia*
Cholesterol	94.33±2.18	97.33±0.88 ns	94.00±1.73 ns
Triglycerides	112.66±2.18	115.33±1.20 ns	106.66±0.88 ns
HDL	29.66±2.33	27.66±0.88 ns	28.00±2.08 ns
LDL	75.66±2.60	73.33±1.45 ns	77.33±1.20 ns
VLDL	29.33±4.05	25.33±1.76 ns	23.66±1.45 ns

Values are expressed as means ± SEM, (n = 5), *** P< 0.001 and ns (non – significant) in comparison to control group.

Table 9. Effect of *Marsilea quadrifolia* and *Salvinia molesta* on liver and kidney function test (Acute toxicity study) 2000 mg/10 ml

Name of tests	Control Group	*Salvinia molesta*	*Marsilea quadrifolia*
Bilirubin (Total)	0.37±0.012	0.35±0.020 ns	0.313±0.009 ns
AST	84.66±2.40	96.66±1.20 *	75.667±0.882 *
ALT	74.33±2.18	83.33±1.45 *	65.66±1.76 *
Alkaline Phosphatase	504.66±2.9	534.66±2.9 *	431.33±1.33 ns
Blood Urea	17.66±0.66	17.33±0.88 ns	16.33±0.88 ns
Serum Creatinine	0.54±0.021	0.52±0.009 ns	0.46±0.015 ns
Serum Uric Acid	3.53±0.02	4.33±0.176 ns	3.53±1.32 ns

Values are expressed as means ± SEM, (n = 5), *** P< 0.001 and ns (non – significant) in comparison to control group.

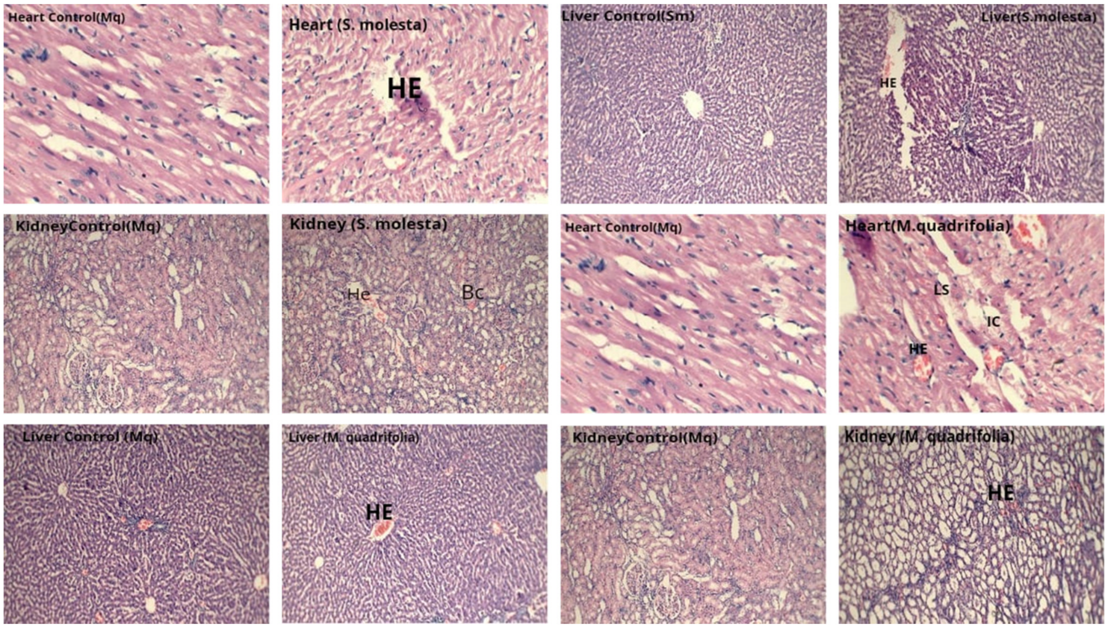

Figure 6. Histopathological examination of heart, liver and kidney of control group and treatment groups, *M. quadrifolia* and *S. molesta* (Toxicity group 2000 mg/kg). Mq (*Marsilea quadrifolia*), Sm (*Salvinia molesta*), FN: focal necrosis, HE: haemorrhage, HY: hypertrophy of epithelial cells, BC; blood cells, IN: acute inflammation, LS: loss of striation, IC: inflammatory cells infiltration, N: necrosis

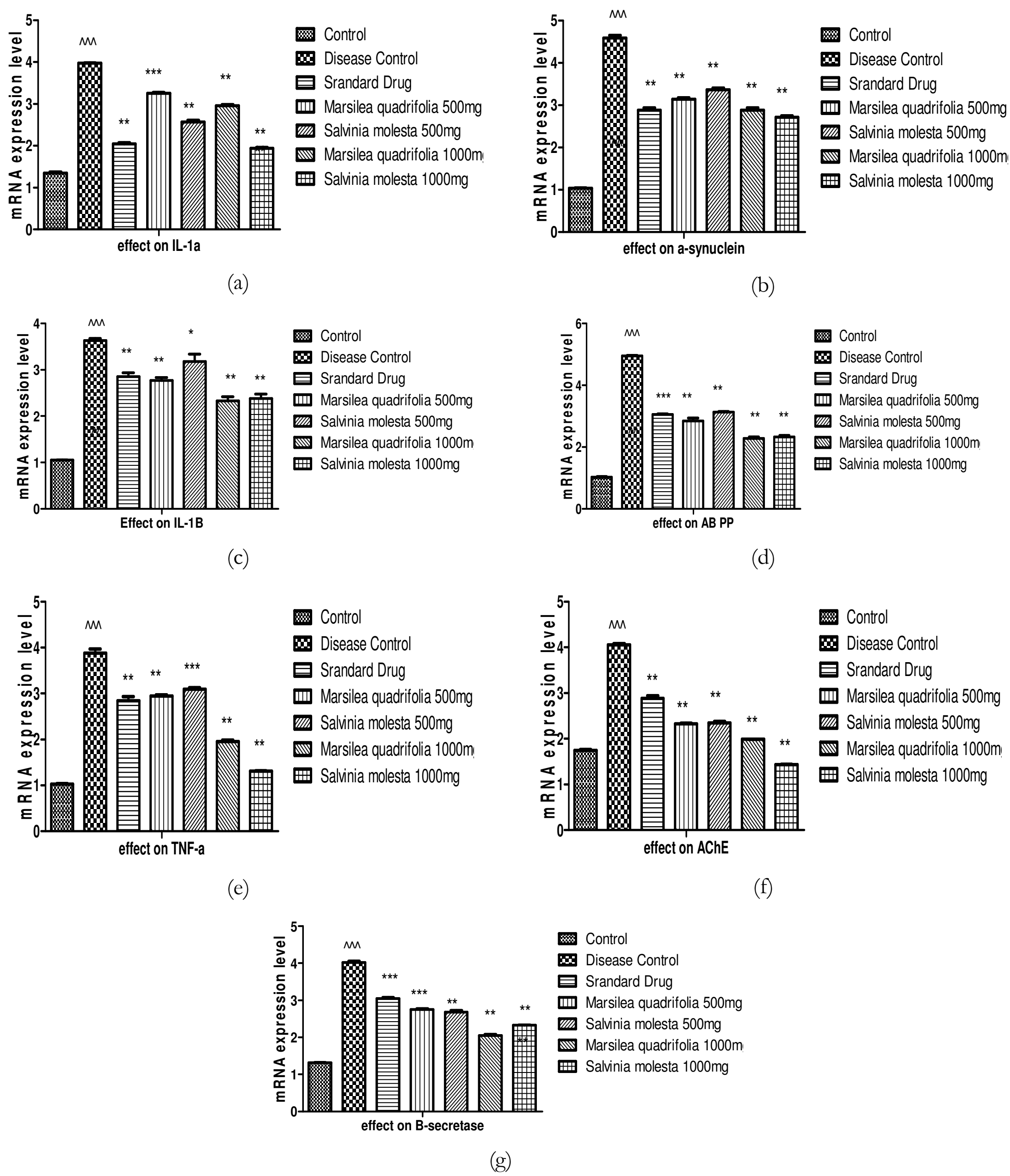

Figure 7. Effect of *M. quadrifolia* and *S. molesta* on various genes of mRNA expression in the aluminium chloride-induced AD model (a,b,c,d,e,f,g). $^{\#\#\#}$ $P < 0.05$ and *** $P < 0.05$ in contrast to control and disease control group respectively

Discussion

The deficiency of AChE is one of features of AD and responsible for most of its symptoms, such as a decline in memory and cognition. AChE inhibitors such as tacrine, donepezil, rivastigmine, and galantamine are currently used as anti-AD drugs (Lakshmi *et al.*, 2020). The side effects of these anti-AChE drugs, such as toxicity, tolerability, and loss of efficiency, have interested the researchers to consider alternative natural anti-AD substances in place of current synthetic medications.

This study revealed the anti-Alzheimer effects of the Ethanolic extracts of *M. quadrifolia* and *S. molesta* plants. Moreover, the phenolics that existed in the structure of the plant and have the potential to show such kind of effects were also identified. The results of the study showed that both the plants had significant potential in terms of Alzheimer disease. The study highlighted the phenolic content of the plants; yet, apart from the standards used in this study, it is possible to find certain structures, which have not been identified until today in the extracts of *M. quadrifolia* and *S. molesta* plants. Therefore, it is important to specify the structures of secondary metabolites existed in *M. quadrifolia* and *S. molesta* plant by isolating them through the activity- driven isolation method, and to study the structures.

We found favourable number of phenolic compounds (Gallic acid and Hydroxybenzoic acid higher in *M. quadrifolia* and Salicylic acid higher in *S. molesta*) in *M. quadrifolia* and *S. molesta* ethanolic extract. Chemical categorization of *M. quadrifolia* and *S. molesta* shown presence of bioactive phytoconstituents such as: "flavonoids, alkaloids, polyphenols and saponins", responsible for its antioxidant activity. Inhibition of lipid peroxidation determined have harmony to our present work. Many plants have flavonoids and phenolic compounds which have many curative properties such as anticancer, antioxidant, free radical scavenging and anti-inflammatory abilities (Coura *et al.,* 2015). It was estimated that certain compounds present in plant extract have antioxidant potential which convert potassium ferricyanide to potassium ferrocyanide and formulate ferric ferrous complex when treated with ferric chloride. It was evaluated that *M. quadrifolia* and *S. molesta* have antioxidant potential when compared to standard ascorbic acid.

To determine the motor coordination wire hanging test were performed. DC group showed slow movement while hanging similar to effect of *Tribulus terrestris* extract and *Cucurbita pepo* extract (Gauthier *et al*., 2003). Bin and Ter groups showed the improvements of locomotor and neuromuscular strength in wire hanging test.

Animals weight is the direct sign of toxic outcome triggered by material under examination and it is more notable if weight loss becomes greater than 10% of initial weight (Zheleva *et al*., 2012). Constant to earlier studies plants under study both yield vigorous results while acute toxicity study indicating normal metabolism and appetite of animals. Metabolic reactions instigated by toxicants mainly target liver, kidney and heart, therefore vital parameters such as haematological, biochemical, liver and kidney function test were performed to evaluate noxious potential of *M. quadrifolia* and *S. molesta* on physiological status of animal.

Haematological investigation demonstrated the safer capacity of both plants on the level of haemoglobin, TLC, RBC, MCH, HCT, MCV, MCHC, lymphocytes, neutrophils, eosinophils and monocytes. While *M. quadrifolia* showed more values of these parameters with respect to *S. molesta.* The amplification in platelet amount representing *M. quadrifolia* and *S. molesta* efficiency in wound healing activity (Saleem *et al.*, 2019). A decrease in cholesterol level upon handling with *M. quadrifolia* and *S. molesta* demonstrating its therapeutic action for plaque development in heart attack, arteries and stroke chances. Behavioural studies were done to assess the neuroprotective effect of *M. quadrifolia* and *S. molesta*. Morris water maze test results of improved latency in *M. quadrifolia* and *S. molesta* treated groups and increased escape latency in disease control group were congruent with preceding reports. In open field test of exploration, locomotion and learning results were compatible with earlier study (Thanabhorn *et al.*, 2006). In Y- maze task it was explored that continued cognizance, short term and distinctive ability of rodents to replacement the neophilia or arms was diminished by prolonged administration of aluminium chloride. Both plants treated groups showed the enhanced cognitive behaviour.

An analysis of (Vaghasiya *et al.*, 2011) documented with our results of Y-maze task which designated the upgraded continued cognition, short term and distinctive ability of rodents to replacement the neophilia or arms was diminished by prolonged administration of aluminium chloride. Our work recommended that

Both plants ingestion increase the muscular strength in rats as it has versatile pharmacological activities. Retention memory discrepancy was examined in passive avoidance test and disease control group showed the worse effect of aluminium chloride on retention memory with decreased step over insensitivity and latency to electric shock and results were demonstrated by earlier study of Lakshmi (Li *et al.*, 2008).

Our outcomes of hole board apparatus for evaluation of neophilia, exploratory and curiosity behaviour were symphonic with findings of (Petrasek *et al.*, 2018). Acetylcholinesterase enzyme triggers hydrolysis of acetylcholine to acetyl- COA and choline. Treating with Aluminium improved the level of acetylcholinesterase enzyme. We assessed in our study that level of acetylcholinesterase was considerably decreased in disease control group and improved in *M. quadrifolia* and *S. molesta* extract treated groups.

Our findings were in agreement with former conclusions as cure with aluminium chloride in disease control group cause the noteworthy decline in the level of first line agent of protective antioxidant enzymes and improved the level of malondialdehyde. The levels of CAT, SOD and GSH were recovered with *M. quadrifolia* and *S. molesta* treated groups and MDA level was evidently condensed due to antioxidant potential of both plants. It was stated that myocardial injury and neurodegeneration was related with reduced level of SOD (Lakshmi *et al.,* 2015). In former studies, it was declared that many mental disorders were related with shortage of CA. GSH is an additional antioxidant enzyme which act as scavenger of hydroxyl radicals, as pool of decreased glutathione and singlet oxygen. Aging process and Neurodegeneration are related with reduced level of GSH. First line protection antioxidant enzymes have essential role in defence against Reactive Oxygen Species (ROS), superoxide radicals and singlet oxygen.

This study directed that *M. quadrifolia* and *S. molesta* have neuroprotective properties in AD model by improving behavioural and biochemical factors. Histopathological analysis also recommended that ingesting of *M. quadrifolia* and *S. molesta* improved the neurofibrillary tangles, neuronic loss, pigmentation and neuroinflammation provoked by aluminium chloride. In this study we determined that *M. quadrifolia* and *S. molesta* in future studies will be helpful in curative methodologies to cope with neurodegenerative ailments owing to its antioxidant potential.

Outcomes of current study discovered that the acetylcholinesterase level in mRNA expression (accountable for motor and neuro muscular functions) was controlled due to aluminium chloride's neurotoxic effect in model group associated to control group maintained by decreased grip strength and motor coordination in neurobehavioral study. Down regulation of AChE related to cholinergic neuronal cell death, apoptosis, senile plaque formations and cognitive disabilities as formerly described (Lakshmi *et al.,* 2015). Although *M. quadrifolia* and *S. molesta* reduced the aluminium chloride's neurotoxic effect on mRNA expression in treatment groups of 1000 mg/kg >500 mg/kg demonstrating defensive effect of both plants on enzymatic features of AChE splice variant. Studies of acetylcholinesterase level decrease in aluminium chloride model group in this study are supported by earlier studies (Asaduzzaman *et al.*, 2010).

Occurrence of neurofibrillary tangles and amyloid plaques are morbid signs of AD. In early phases of AD proteolytic cleavage of β-site APP by BACE1 triggered the development of β-amyloid ß peptides. BACE1 has a mandatory role in assembly of β-amyloid peptides and its removal completed the β-amyloid peptides generation (Santambrogio *et al*., 2015). Impairment of synaptic function and Aβ peptide accumulation directly involved this enzyme. BACE1 is chiefly expressed in neurons and brain tissues. Therefore, BACE1chemical inhibition is measured as disease altering therapeutic object in early Phases of AD. In former human trials, and animal studies and it was authenticated that BACE 1 inhibition yield useful therapeutic effects on Cognition and memory (Parihar and Hemnani, 2003). The level of BACE1 expression was adjusted in aluminium chloride induced model group but *M. quadrifolia* and *S. molesta* suggestively repair the promotion of BACE1 level in 1000 mg/kg of both plants treated group representative its possible curative effect consistent to previous findings (Zaky *et al.*, 2017).

Tumour necrosis factor alpha (TNF-α.) a principle pro-inflammatory cytokine involved in neuroinflammation via increasing expression of BACE1 accountable for intensified Aβ increase and tau hyper phosphorylation which are main pathological symbol of AD. It was described that inhibition of TNF-α by anti-inflammatory agents have therapeutic potential against cognitive incapacities in AD rodent models (Wang *et al.*, 2001). In clinical trials phase I and phase II, it was assessed that inhibition of TNF-α by anti-inflammatory agents decreased the cognitive diminution and improve daily actions in AD patients. In this study we discovered that aluminium chloride amplified the countenance of TNF-α in model group by triggering proinflammatory cytokines involved in neuroinflammation well-matched to earlier results of (Das and Yan, 2017) *M. quadrifolia* and *S. molesta* treated group showed the decrease in mRNA expression of TNF-α in contrast to model group which designated its modulatory role in TNF-α signalling, motor and cognitive function, therefore its useful role in AD supported by earlier conclusions (Vassar *et al., 2014,* Decourt *et al.* 2017).

Neurodegenerative diseases like AD are related to elevated level of pro-inflammatory cytokines interleukins IL-1α and IL-1β in the surroundings of amyloid plaques accountable for provoking neuroinflammation. (Parihar and Hemnani, 2003). The elevated level of IL-1 performing a central role in pathogenesis of AD as it raised level has been noticed in PD in AD and CSF patients. Stimulated microglial cells cause IL-1α and IL-1β activation which brought the release of multitude representing molecules like chemokines, adhesion molecules, cytokines and prostaglandins mainly TNF-α and IL-6 from neuroglial and endothelial cells in the progress of neurodegeneration. It was described in earlier findings that rats' brain inability to IL-1β even a single bolus injection triggered rapid and strong activation of microglial cells and astrocytes which raised the appearance of pro-inflammatory cytokines (Shi *et al.,* 2011). It was described in previous findings that IL-1β is capable to activate its own expression and playing principal role in neuroinflammation related with reduction in learning and memory capabilities. Our results of decreased learning and hippocampal supported memory capacities in aluminium chloride induced model group due to raised expression of IL-1α and IL-1β. The previous studies representing that IL-1β raised expression is connected with clogged long-term potentiation (LTP) related to hippocampal dependent memory and learning supported our current results (Depino *et al.*, 2005). It was determined in the investigational methodology of (Shaftel *et al.*, 2008) that the raised level of interleukin IL-1α and IL-1β through the process of neurodegeneration in AD cause cognitive, evocation and intellect disfunction.

This study indicating that the consumption of aluminium chloride induces neuronal insult causing in accumulation of amyloid plaques which may motivated a self - endorsing cytokine cycle responsible for triggering violent response loop of continuous IL-1α and IL-1β propagation encouraging progression in neuronal and synaptic disfunctions supported by earlier works (Craft *et al.*, 2005). In former tissue culture findings, it is described that IL-1α and IL-1β improved the mRNA expression of gamma secretase and β-APP activity more involved in pathogenesis of AD, proposing that the raised mRNA expression of IL-1α and IL-1β are likely offender of neurodegeneration (Muhammad *et al.,* 2020). However, *M. quadrifolia* and *S. molesta* 1000 mg/kg reduced the elevated level of mRNA expression of IL-1α and IL-1β showing its curative prospective to decrease neuroinflammation and its defensive role in memory association.

Conclusions

Both plants showed inhibitory activity against the enzyme acetylcholinesterase and they also proved to be potent antioxidant. *M. quadrifolia* and *S. molesta* showed high values for both assays. The ethanolic extract of *M. quadrifolia* showed the best inhibition of AChE and a very good antioxidant activity as well. *S. molesta* also proved to be a good inhibitor of acetyl cholinesterase whereas, *S. molesta* inhibited to less extent. *M. quadrifolia* and *S. molesta* may help in preventing AD as they showed inhibitory activity against AChE and at the same time both bear good antioxidant potency. Finally, these plant extracts and their active components could emerge as natural antioxidants, alternative anticholinesterase drugs and can serve as starting points for synthesizing more effective AChE inhibitors.

Authors' Contributions

Conceptualization: UH; Data curation; UH, SM; Formal analysis; ZC, MAS; Investigation; UH, ZC, MAS; Methodology; MAS; SM; Project administration; ZC; Resources; MQ; Software; MM; Supervision; UH, MAS; Validation; MD; Visualization; MD; Writing - original draft; SM; Writing - review and editing SM.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Ref. No. GCUF/ERC/03.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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