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Fucoidan serves a neuroprotective effect in an Alzheimer's disease model

Mamangam Subaraja[1,2], Dhanabalan Anantha Krishnan[3], Varghese Edwin Hillary[1], Tharsius Raja William Raja[1], Pratheesh Mathew[1,4], Sundaram RaviKumar[5], Michael Gabriel Paulraj[1], Savarimuthu Ignacimuthu[1, 6]

[1]Division of vector control, Entomology Research Institute, Loyola College, Chennai-600034, Tamil Nadu, India, [2]Department of Biochemistry, Vivekanandha College of Arts and Sciences for Women (Autonomous), Tiruchengode - 637 205, Tamil Nadu, India, [3]Center of Advanced Study in Crystallography and Biophysics, University of Madras, Chennai-600 025, Tamil Nadu, India, [4]Department of Zoology, St. Thomas College, Kottayam-686 574, Kerala, India, [5]Department of Biomedical Science, Alagappa University, Karaikudi-630 003, Tamil Nadu, India,[6]Xavier Research Foundation, St. Xavier's College, Palayakottai-627001, Tamil Nadu, India

TABLE OF CONTENT

1. ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that causes memory and cognitive deficits. The present study was carried out to evaluate the protective effects of fucoidan in monocrotophos induced AD in *Drosophila melanogaster*. *In silico* studies showed that fucoidan exhibited binding energy of -9.3 kcal with proteins. Consistent with this, fucoidan, in a dose and time-dependent fashion, had inhibitory activity against cholinergic and monoamine-metabolized enzymes *in vitro*. Fucoidan inhibited the increase in total *mRNA* and protein in monocrotophos fed flies and prevented changes in biochemicals, neurochemicals and latency time of locomotor, learning and memory induced by monocrotophos. Together, the findings show that fucoidan serves a neuroprotective effect in Alzheimer's disease model in *D. melanogaster.*

2. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease (NDD) which causes memory and cognitive decline, *i.e.,* dementia, decreased physical function leading to patient's death. It is estimated that 4.7 million people are living with AD; it is expected to affect one in 85 people globally by 2050 (1, 2). Numerous diverse hallmarks of pathology have been reported such as accumulation of β-amyloid, hyperphosphorylated tau protein, and reduction of acetylcholine, oxidative stress and dyshomeostasis of biometals in neurons *etc,* (3). Monocrotophos is a well-known toxin and people are frequently exposed to monocrotophos such as gardeners and farmers who suffer from AD (4); 50% of the workers had neurological symptoms such as permanent neuron damage, rigidity muscle, auditory, vestibular dysfunction, weakness, loss of reflexes and easy fatigability (5). Aβ peptides modify PI3K/AKT/mTOR/MAPK/ERK signaling pathway (also known as the Ras-Raf-MEK-ERK pathway), cyclin-dependent kinase 5 (cdk5), and tropomyos in receptor kinase A (TrkA) leading to NDD (6). It has also been demonstrated that accumulation of Aβ led to the development of oxidative stress, disturbed metal ion homeostasis, Ca^{2+}dysregulation, cholesterol dyshomeostasis, impaired neurotransmissions, mitochondria and endoplasmic reticulum dysfunction (7). Insect's biogenic amines are arylalkylamines, dopamine, tyramine, serotonin, and octopamine etc., and they act as neurotransmitters (NTs), neuromodulators, or neurohormones and also are involved in motor and cognitive behaviours (8). Inhibitory NTs (GABA and glycine) and exhibitory NTs (glutamine and acetylcholine) cause imbalance in NDD patients (9).Fruit fly genome is well documented; it is related to 75% of human genes that are associated with NDD (10). So, researchers use *Drosophila melanogaster* as model organism for neuroscience and neuronal behaviours studies because of its genetic tractability, complex behavior, neuroanatomy and orthologous nature to human genes (11). Pharmacotherapy for AD currently deals with symptomatic relief and regulated targets at cognitive revival (12). For the past few decades, drugs were extensively screened to treat AD; they were found beneficial without any side effects. Manzamine–A is a glycogen synthase kinase 3 (GSK 3)/CDK5 inhibitor and reduces tau phosphorylation (13). The current study evaluated the effect of fucoidan on neuronal behaviour and neuropathological changes in monocrotophos induced AD in *D. melanogater*.

3. MATERIALS AND METHODS

3.1. In silico studies

3.1.1. Protein preparation

Molecular docking studies were carried out using various test compounds with proteins such as acetyl cholinesterase (PDB ID: 4M0E), amyloid-beta (PDB ID: 2BEG), BACE1, (PDB ID: 5NN8), Tau/ GSK -3β (PDB ID: 1J1B), TrkA ,(PDB ID: 4PMS) and phosphoinositide 3-kinase (PI3K; PDB ID: 5JHA). Missing hydrogen atoms were added and correct bond orders were assigned, and then formal charges and orientations were fixed. The amino acid flips were assigned and H-bonds were optimized. Non-hydrogen atoms were minimized; the root mean square (RMS) reached default value of 0.3 Å. Sitemap 2.3 was used to investigate the binding site in the docking studies.

3.1.2. Ligand preparation

The test compounds were built using builder panel in Maestro. The compounds were taken for ligand preparation by Ligprep 2.3 module (14) which performed addition of hydrogen, 2D to 3D conversion, realistic bond lengths and bond angles, low energy structure with correct chiralities, ionization states, tautomers, stereo chemistries and ring conformations.

3.1.3. Induced fit docking

Induced fit docking (IFD) was studied by the method of Trott *et al.*, (15). The prepared protein was loaded in the workspace and the sitemap predicted active site was specified for IFD. The grid was calculated for about 20 Å to cover the active site residues. The van der Waal's radii of the non- polar receptor and ligand atoms were scaled by a default factor of 0.50. IFD was calculated for the test compounds against AD drug targets. 20 conformational poses were calculated where the best conformational pose was selected based on the docking score, glide energy, hydrogen bonding and hydrophobic bonding interactions.

3.2. *In vitro* studies

3.2.1. Maintenance of cell culture

The PC12 cells were purchased from National Centre for Cell Science (NCCS), Pune and grown on poly styrene tissue-culture dishes Dulbecco's modified Eagle's medium (DMEM) containing 10% of foetal bovine serum, 5% of horse serum supplemented with 1mM of pyruvate, 2mM of glutamine, 100 units/ml of penicillin, and 100 units/ml of streptomycin with 90 % of air, and 5% of CO_2. Cells were sub-cultured in 96-well plates at a cellular density of 10^4 cells per well and allowed to adhere and grow. When cells reached the 95% of confluence, they were placed in serum-free medium.

3.2.2. Kinetic study of cholinesterase inhibition in PC12 cells

The inhibition of AChE and BuChE were assessed by using the method of Ellman *et al.*, (18). The 96-well plates were incubated with 0.01ml of different concentrations (0.001 to 100µM) of test compounds at 37 ^oC for 6min, followed by the addition of to 0.03 ml of acetylthiocholine iodide or butyrylthiocholine iodide. Kinetic characterization of the hydrolysis of ACh and BuCh catalyzed by cholinesterase was performed. The absorbance was read at different time intervals (0.0, 70, 140, and 210 s) at 405nm. The concentration of test compounds that produced 50% inhibition (IC_{50}) of cholinesterase activities was calculated by nonlinear regression analysis of the response-concentration (log) curve.

3.2.3. Kinetic of monoamine oxidase (MAO) A and B in PC12 cells

To study the MAOs inhibition, briefly, the five different concentrations (0.0, 0.120, 0.320, 0.620µg) of 0.1 ml of test compounds and 0.9ml of MAO were incubated at 37 ^oC for different time intervals (0, 15, 30, 60min) in 96-well microplate. The substrate concentrations (0.03, 0.1, 0.23, 0.36, 0.5, and 1.0µg) of p-tyramine were used and the initial catalytic rates of MAO were estimated in the presence and absence of different concentrations of test compounds. The reaction was started by adding 0.1 ml of Amplex Red reagent, 0.2 ml of horseradish peroxidase, and 0.5 ml of p-tyramine and pargyline for 20 min at 37 ^oC. The H_2O_2 production was read at 590nm. The MAO inhibition was estimated by constructing the Liner weaver-Burk plots and the kinetic data were expressed in K_m and V_{max} values.

3.2.4. Cell viability assay

Cell viability was measured by the method of Mosmann (16) using the MTT reagent. The toxicity effects of test compounds on PC12 cells were evaluated. Briefly, cells in 24-well plates were incubated with different concentrations of test compounds for 24 h and 48 h. The cells were rinsed with 1.0 ml of 1M PBS (pH 7.4); 0. 2 ml of MTT was added to wells and incubated for 30 min at 37°C. After removing the medium with MTT, living cells containing MTT formazan crystals were solubilized with 0.2ml of DMSO and read at 570 nm.

3.2.5. Differentiation of PC12 using different concentrations of fucoidan for 7 days

Cells were seeded (10^4 cells/ cm^2) in 6-well plates and after 80% confluence was attained, fucoidan was added in test groups at concentrations ranging from 5 to 100 µg in DMEM and maintained in CO_2 incubator. The cells which did not receive fucoidan served as control and 0.1 % of vehicle controls were treated alone.

After incubation with fucoidan at the end of experimental period, cells were washed with medium and further studies were carried out.

3.2.6. Blood brain barrier (BBB) permeation assay

BBB permeation assay was assessed by the method of Pardridge (17). Brain penetrations of test compounds were evaluated using a parallel artificial membrane permeation assay (PAMPA). The donor 96-well microplate (PVDF membrane pore size 0.45 µm, diam. 25 mm) and the acceptor microplate were both from Millipore (Sigma-Aldrich). The acceptor microplates (Costar®) were filled with 0.3 ml of PBS: ethanol (7:3), and the filter membranes were impregnated with 0.004ml of porcine brain lipid (PBL) in dodecane. Test compounds at the concentrations of 0.2 ml were added to the donor wells. The acceptor filter plates were placed on the donor plates and incubated for 16 hours at 25°C (degree Celsius). After incubation, the donor plates were removed and the concentration of test compounds in the acceptor wells were estimated using a UV plate reader (FlexStation®3). Samples were analyzed at five wavelengths and the results were tabulated.

3.2.7. Neurite length assay

To analyze the percentage of neurite length, cells were observed using a light microscope and images captured using a camera connected to the microscope at 50x. 50 % of cells per field were counted and the neurite length was estimated using Magnus Pro software on the images. The µm of neurite lengths was converted into percentage of neurite length. The percentage of neurite length was calculated as the total of neurite length cells divided by the number of cells multiplied by 100

3.3. *In vivo* studies

3.3.1. Ethical statement

D. melanogaster is 'non-regulated insect model and therefore this research did not require the approval of our Institutional Animal Ethical Committee.

3.3.2. Preparation of fly food

Drosophila melanogaster diet was prepared from 100 ml of semi-solid diet which contained 5.5 gm of corn flour, 3.5 gm of sucrose, 2.0 gm of dextrose, 1.5 gm of agar, 1.0 ml of propionic acid and 1.0 g of yeast extract. After preparation, flies were transferred to Erlenmeyer flask containing media.

3.3.3. Maintenance of *D. melanogater*

Drosophila melanogater (adult male flies, red eye) was authenticated and stock cultures were maintained at Entomology Research Institute (ERI), Loyola College, Chennai, Tamil Nadu, India. The flies were maintained at 25^{o}C (80 % relative humidity) with 12:12 hours of light and dark cycle.

3.3.4. Experimental design

Flies were divided into six experimental groups as given below. The total number of flies in each group (n) was 50.

Group 1: Served as control *D. melanogaster*
Group 2: *D. melanogaster* exposed to 25 µg of monocrotophos
Group 3: *D. melanogaster* exposed to 25 µg monocrotophos plus50 µg fucoidan
Group 4: *D. melanogaster* exposed to 50 µg of fucoidan
Group 5: *D. melanogaster* exposed to 50 µg of donepezile

After 10 day period, behavioural studies were analyzed; then their brains were dissected, and further studies were carried out.

3.3.5. Food intake tracer method

Quantification of food intake was studied by the method of Ja *et al.*, (19) using *Drosophila melanogaster*. Adult flies (20/vial) were placed in food vials and one capillary feeding tube per vial by a plastic pipette tip (5 mm) was placed. Flies consumed liquid medium (5% sucrose and 1% blue 1dye in water) from the capillary tubes for 8h; the amount of liquid medium consumed from each tube was recorded, and flies were then provided with fresh capillary feeding tubes containing 5% sucrose medium without blue dye for 18h. Flies were housed in the same vials while consuming medium from both the first (containing blue dye) and second (without blue dye 1) capillary tubes. Excreted blue dye of vials was collected by adding 3ml of water to each group

of insects followed by vortexing. The optical density of dye was read at 630nm and absorbance values were converted to volumes by interpolation from standard curves of dye. Vials without flies were used to control evaporation of liquid medium from capillary tubes. The descent of the top meniscus of liquid was monitored for 24 hours and the volume consumed was expressed in units of µl per fly.

3.3.6. Dissection of *D. melanogaster* brains

D. melanogaster were anesthetized at-40 °C and brains were dissected by using dissection microscopy. The heads were decapitated and placed in a glass dissection dish well with 1.5 ml of 1M phosphate buffer saline (pH, 7.4). The petri dish was coated with sylgard transparent resin containing drops of PBS. The head was held and the maxillary palps were removed on both sides with two pairs of forceps to open the head cuticle. Then the brain was carefully removed from the head cuticle and excess surrounding tissue was removed using fine mounted pin and the brain lobes were stored at -80 ºC.

3.3.7. The extracts of proteins in brain

The brain tissues were removed and 100 mg of tissues were homogenized with 1.0 of 1mM PBS using pestle and mortor at -4ºC. The cells were pelleted by centrifuging at 12,000rpm for 10 min and the supernatant was centrifuged at 10,000rpm for 15 min. The supernatant was used for ELISA analysis.

3.3.8. Estimation of AChE, PKI3, TrkA, Tau, BAECl, APP

The levels of AChE, PKI3, TrkA, Tau, BAECl and APP were quantified using a sandwich enzyme-linked immunosorbent assay. The absorbance was determined at 450 nm using Spectra Max M3 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, United States) in accordance with the manufacturer's instructions. All experiments were performed in triplicate.

3.3.9. Extraction and quantification of glutamate and gamma –aminobutyric acid (γ-GABA) contents

The contents of glutamate and GABA were estimated using paper chromatographic method of Papastamatis and Wilkinson, (24). 50 mg of brain were homogenized with l ml of 0.3 M triethanolamine buffer (pH 6.8) and then centrifuged at 15,000rpm for 20 min. The extract was re-suspended in 1.0 ml of 20 mM phosphate buffer saline (pH 7.4) and centrifuged at 10,000rpm for 10 min. The extraction was spotted on Whatman filter paper (No. 1) by using solvent phenol: H_2O system (80:20). The paper was air tired. GABA and glutamate were located by ninhydrin spray. GABA and glutamate spots were cut out, eluted and were quantified.

3.3.10. Extraction of brain biogenic amine

5mg of brain was dissected and homogenized with 0.5 ml of 0.1 M perchloric acid. After centrifugation at 15,000rpm for 30 min, the supernatant was collected. Biogenic amines were measured using paper chromatography. The levels of serotonin, dopamine, tryptamine and octopamine were estimated by the method of Erspamer and Boretti (25). The activities of AChE and BuchE were assessed by using the method of Ellman *et al.*, (18).

3.4. Estimation of oxidative stress indicators

3.4.1. Determination of lipid peroxidation (LPO)

The formation of thiobarbituric acid reactive substances (TBARS) was estimated by the method of Beige and Aust (20). In brief, the brains were homogenized with 0.5 ml of 15% trichloroacetic acid (TCA), 0.2ml of 0.35% tert-butyl alcohol (TBA) and 0.1 ml of 5N of hydrochloric acid (HCl); it was incubated at 95 °C for 15 min. The supernatant was kept for 20 min at 37ºC and centrifuged at 12,000 rpm for 5 min. The absorbance was read at 535 nm. The content of lipid peroxidation was expressed as a TBARS produced in nanomoles per mg of protein.

3.4.2. Determination of catalase (CAT) activity

The activity of CAT was estimated by the method of Yumoto *et al.*, (21). 1 ml of 50 mM phosphate buffer (pH, 7.4) containing 0.1 ml of 100 mMH_2O_2 was added to 0.1 ml of homogenized brain. It was incubated for 2 min at 37 °C and absorbance was read at 240 nm. The CAT activities were expressed in units per mg protein, and one unit of enzyme activity was defined as the amount of enzyme required to break down 1 mM of H_2O_2.

3.4.3. Determination of superoxide dismutase (SOD) activity

The activity of SOD was determined by the method of Marklund and Marklund (22) using the auto-oxidation of pyrogallol. Briefly, 1 ml of 62.5 mM tris–cacodylic acid buffer and 0.5 ml of 4 mM pyrogallol were added to 0.1 ml of homogenized brain. To observe the auto-oxidation of pyrogallol, the absorbance was measured at 420 nm.

3.4.4. Determination of reduced glutathione (GSH) levels

The levels of GSH were estimated by the method of Moron *et al.*, (23). 0.1 ml of homogenized brain was treated with 0.1 ml of 25% TCA and the precipitate was pelleted by centrifuging at 70, 000rpm for 10 min. The free endogenous sulfhydryl group was estimated in 3.0 ml of a reaction mixture containing 2 ml of 0.5 mM 5, 5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and 1.0 ml of tissue supernatant. The GSH reacted with DTNB and absorbance was read at 412 nm.

3.4.5. Estimation of immunomodulatory molecules

The levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were analyzed using ELISA kits (Molecular Devices Corporation, Sunnyvale, CA, United States) in accordance with the manufacturer's instructions. The absorbance was determined at 520 nm using Spectra Max M3 microplate reader. All experiments were performed in triplicate.

3.4.6. Estimation of apoptotic regulator molecules

The levels of B cell lymphoma 2 (Bcl-2) and caspase-3 (Casp-3) were analyzed using ELISA kits (Molecular Devices Corporation, Sunnyvale, CA, United States) in accordance with the manufacturer's instructions. The absorbance was determined at 470 nm using Spectra Max M3 microplate reader. All experiments were performed in triplicate.

3.4.7. Isolation of RNA and cDNA synthesis

RNA samples were isolated from brain of flies using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA from brains was extracted using the Trizole reagent (Gibco). RNA was converted to cDNA using oligo (dT) and Superscript II reverse transcriptase (Invitrogen).

3.4.8. Quantitative real time -PCR (qRT-PCR)

qRT-PCR was performed using real-time PCR system with SYBR Green PCR Master Mix (Takara's). Specific primers were used (Table 1). PCRs were denatured at 94°C for 5 min, followed by a 29-s denaturation at 94°C, annealing at 55°C for 33 s, and an extension at 72°C for 1min. Threshold cycle was used to assess relative levels of m-RNAs versus reference beta-actin. Quantification was performed in two independent experiments with five samples for each group set up in triplicates.

3.4.9. Semi-Qualitative RT PCR

Qualitative RT-PCR was done using specific primers (Table 1). The PCR mixture contained 200 nM of reverse and forward primers with about 500 ng of cDNA. PCRs were denatured at 94°C for 3 min, annealed at 55°C for 30 s, and extended at 72°C for 2 min. Products were loaded and electrophoresed in 2 % agarose gel.

3.5. Neuronal behaviour analysis

3.5.1. Climbing analysis

The behaviour was assessed by the method of Pendleton *et al.*, (26) for locomotor ability in *D. melanogater*. Fifty flies were placed in 100 ml measuring cylinder and allowed 10 min to acclimatize. During the test, fifty flies of control and experimental groups were placed in the far left bottom tube at the loaded position. Flies were gently tapped down to the bottom of the tube three times with a force of one tap per second. The top receiver tubes were quickly moved to the left, allowing flies 20 sec to climb up into the top receiver tube from a started position in each trial. Flies that succeeded in climbing up to the receiver tube were transferred to the next bottom tube at the end of the 20 sec by moving the array of top tubes one slot to the right, followed by gentle tapping and prompt placement of top tubes back to the starting position. The flies were tapped down and allowed to climb up for 20 s and then tapped down again. This was recorded and repeated three more times. At the fourth time, the

Table 1. Primers used for the experiments

Gene ID	Genes	Primers	Tm	Product length
NM_001170129.2	Acetylcholine esterase transcript variant B (Ace)	Forward primer- GTCACGCTGGTTCCGATTTT Reverse primer-TCGGTCGCTCACTACATGAC	59	143
NM_170146.2	Nicotinic acetylcholine receptor alpha2, transcript variant B (nAChRalpha2)	Forward primer-GCAGCGAGACTTGGAGACTT Reverse primer-GACCTTGACGCTTTCTGCCC	60	139
NM_001258519.1	Beta amyloid protein precursor-like (Appl), transcript variant B	Forward primer- CGAGAACCAGCGACAAAGTG Reverse primer-TCCTGGTCCTATCTCCTGGC	59	142
AY032977.1	Microtubule-associated tau protein	Forward primer- GGACTTCAAGGACAAGGCCA Reverse primer-TGGATCTTGATGTCTCCGCC	55	84
NM_168856.3	Presenilin, transcript variant B (Psn)	Forward primer- TTTCCTTAACTGGGGAGGCG Reverse primer-GAGCACGAAGCCTGGAGATT	55	88
NM_078497.4	Actin 5C, transcript variant B (Act5C)	Forward primer- -TCGCCACTTGCGTTTACAGT Reverse primer-AGCATCGTCTCCGGCAAATC	60	147
NM_001273974.1	Transcript variant B (Mtor)	Forward primer- ATTCTCCAGCTTGCGAGTCC Reverse primer-CCACTTTGTAGTGCTGGGGT	60	177
NT_033778.4	Vesicular monoamine transporter (Vmt)	Forward primer-CTCATCTCGCACTGAACGCA Reverse primer-CAGGCAGCAGACAACAAGTG	60	191
NT_033778.4	Serotonin transporter (SerT)	Forward primer-CCTGCTCTTCACTGCTCCTC Reverse primer-TGCTTAACTCCGGTTGCAGT	60	165

number of flies passing the tube (16.5 cm) was recorded. The percentage of flies that escaped was then calculated.

3.5.2. *T Maze* analysis

T Maze test was done by using the method of Versace and Rosenberger, (27). *T Maze* (31 × 17.5 cm) consisted of starting chamber and a central chamber (12 × 8 × 1.5 cm) connected on each side to a food chamber. The starting chamber (9.5 × 2.5 cm) contained the flies at the beginning of each time slot. Food chambers (9.5 × 2.5 cm) were filled with 4 ml of food. In each experimental phase, flies began the exploration of the apparatus from the starting chamber. The central chambers were connected to the food chambers with a funnel that prevented flies to re-enter the central chamber. A performance index (PI) was calculated using the following formula: The percentage distribution (%) for chamber = Number of flies in the chamber / Numbers of flies per test ×100.

3.5.3. *T Maze* analysis

T Maze test was done using the method of Simonnet *et al.*, (28) with minor modification. The plastic *T Maze* consisted of three chambers. The control chamber was (13.5 x 9.3 x4.5 cm) connected to the left chamber (12 × 8 × 1.5 cm) and the right chamber (12 × 8 × 1.5 cm). The filter papers (6 mm diameter) were soaked in 40 µl of ether and placed in one of the chambers for 24 hours. The number of flies which entered the ether solution for 30 min interval was calculated. The olfactory index was calculated as follows: olfactory index = Number of flies in the without ether chamber - Number of flies in the with ether chamber/Total number of flies.

3.5.4. Aggression analysis

Aggression assays were studied by the method of Hoffman, (29). A customized acrylic was filled with 2% of agar to moisturize the chambers (1 cm wide, 4 cm long and 0.6 cm high). Flies were placed in the center of the spacer and their movements were recorded simultaneously on video and subsequently analyzed for behavior (chasing, touching, wing extension, or copulation). The aggression index (CI) was calculated by dividing the time spent in aggression divided by the total time until aggression.

3.6. Statistical analysis

Data were analyzed using Graph Pad Prism 4.03 software (San Diego, CA). Data were reported as mean ± SEM. One-way analysis of

Table 2. Docking score and inhibition constant of tested compounds with acetyl cholinesterase and amyloid-beta

	Acetyl cholinesterase		Amyloid-beta	
Compounds	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**
Donepezil (3152)	-8.88	85.4	-8.05	65.5
Dermatan sulphate (32756)	-10.03	59.3	-5.74	169.99
Manasamine- A (12020457)	-9.98	60.4	-3.11	435.6
Graciline (15384996)	-11.35	42.4	-1.99	745.5
Graciline 73162010	-12.04	35.7	-2.99	549.4
Fucoidan (92023653)	-8.22	87.9	-8.12	69.3
Macrolecrin-A 73162010	-13.17	39.2	-2.48	694.4

variance (ANOVA) followed by the least significant difference (LSD) tests were done. A value of $p< 0.05$ was considered statistically significant.

4. RESULTS

4.1. *In silico* studies

4.1.1. Binding interactions of test compounds and target proteins

The binding modes of the test compounds with GSK-3β/Tau, A β, AchE, Tau, PIK3, TrkA and BACE1 which are involved in the neuropathology were obtained using molecular docking. Six potential compounds, *i.e* donepezil (3152), dermatansulfate (32756) manasamine-A (12020457), graciline (15384996), galantamine (92023653), fucoidan (92023653), macrolectin-A (92023653) interacted with important catalytic sites of acetylcholine esterase exhibiting good binding energy. The catalytic sites were PHE 295, HIP 447 and SER 203; TYR 72 , THR 75, ARG 296, ASP 74, SER 293 and PHE 295; ARG 296, PHE 295 and GLY122; PHE 295, SER 293, HIS 287, ARG 296; ASP 74 and HIS 447; ASP 74, THR 75, LEU 76, PHE 295 and ARG 296; at a distance of 3.2 Å, 2.8 Å and 3.0 Å; 3.1 Å, 2.7 Å, 3.0 Å, 2.8 Å, 3.0 Å and 3.2 Å; 3.2 Å, 3.2 Å and 3.1 Å; 3.1 Å, 3.2 Å, 2.9 Å and 2.3 Å; 3.1 Å and 3.2 Å; 2.7 Å, 3.0 Å, 2.4 Å, 3.2 Å and 3.0 Å (Table 2 and Figure 1). Amyloid-beta protein bound to dermatan sulphate by interacting with ASP 23, GLY 25 and LYS 28; it bound to manasamine-A by interacting with MET 35; it bound to galantamine by interacting with MET 35; it bound to fucoidan by interacting with ALA 21 and GLU 22; it bound to macolectin- A by interacting with MET 35 and GLU 22. Interactions of hydrogen bonds, distances and binding energy values are shown in Table 3 and Figure 2. BACE1 bound to donepezil by interacting with GLN 73 and THR 72; it bound to dermatan sulphate by interacting with GLY 11, ARG 235, GLN 73, THR 232 and LYS 321; it bound to manasamine –A by interacting with GLY 230; it bound to graciline by interacting with ARG235; it bound to galantamine by interacting with GLY 230, THR 232, GLY 11, GLN 73 and ASP 32; it bound to fucoidan by interacting with ARG 235, THR 72, TYR 198, GLY 34, ARG 128, ASP 32 and GLY 230; it bound to macrolectin -A by interacting with GLN 12 and PHE 108 (Table 4 and Figure 3).

Tau/GSK-3β target protein bound to donepezil by interacting with ARG 220; it bound to dermatan sulphate by interacting with CYS 218, ARG 220, GLN265 and ARG 22; it bound to manasamine-A by interacting with GLY 262; it bound to graciline by interacting with GLY 262; it bound to galantamine by interacting with CYS 218, ARG 223, ILE 228 and TYR 288; it bound to fucoidan by interacting with TYR 288, VAL 263 and GLU 290; it bound to macrolectin-A by interacting with ASN 64, ASP 200 and VAL 135. The glide energy and docking score of tested compounds are given in Table 5 and the interactions are shown in Figure 4. Figure 5 shows the interaction of TrkA target protein with test compounds. TrkA bound to donepezil by interacting with ARG 599 and MET 592; it bound to dermatan sulphate by interacting with GLU 590, ASP

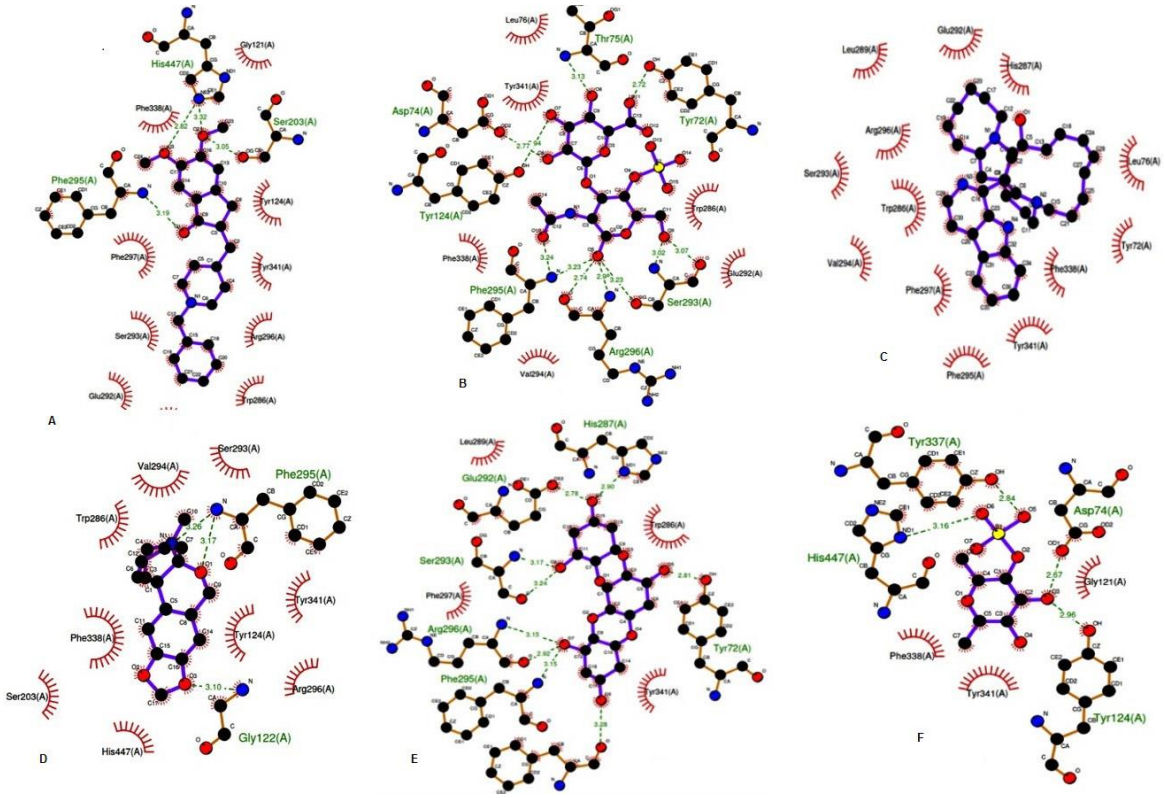

Figure 1. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with AchE.

668, GLU 560, LYS 544, GLY 670 and MET 67; it bound to manasamine-A by interacting with GLY 670; it bound to graciline by interacting with MET 592; it bound to galantamine by interacting with GLU 560; it bound to fucoidan by interacting with MET 671 and ASP 668; it bound to macrolectin- A by interacting with MET 592, TYR 591, ARG 599 and SER 673. Interaction of hydrophobic and glide energy is shown in Table 6. PI3K bound to donepezil by interacting with VAL 882, TYR 867 and SER 806; it bound to dermatan sulphate by interacting with LYS 890, SER 806, LYS 807, LYS 808, LYS 833, ASP 964 and GLU 880; it bound to manasamine- A by interacting with SER 806; it bound to graciline by interacting with THR 887, ASP 950, ASP 964 and SER 806; it bound to galantamine by interacting with THR 887, ASP 950, ASP 964 and SER 806; it bound to fucoidan by interacting with ASP 950, LYS 807, ASN 951, ASP 964, LYS 808 and LYS 833;it bound to macrolectin-A by interacting with ASP 950, ASP 954 and LYS 83 (Figure 6). Interaction of hydrophobic and glide energy is shown in Table 7. Macrolectein interacted with GSK-3β/Tau, A β, AchE, Tau, PIK3, TrkA and BACE1 (Figure 7). Detailed intermolecular interactions and binding energy are given in Tables 8-10. They show the docking results of all target proteins with respect to all the compounds with estimated inhibitor concentration (ki). The above docking results suggested that fucoidan bound to AD target proteins.

4.2. *In vitro* studies

4.2.1. Cytotoxicity in Pheochromocytoma (PC12) cell

The morphological, differentiated and neuritent growth changes of PC12 cells treated with different concentrations of facoidan with monochrotophos for 7 days are given in Figures 8 and 9.

4.2.2. Blood–brain barrier (BBB) permeation

The fucoidan exhibited molecules permeability values of $3.3x10^{-6}cms^{-1}$when compared

Table 3. Docking score and inhibition constant of tested compounds with BACE1 and Tau/3β-GSK

	BACE1		Tau/3β-GSK	
Compounds	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**
Donepezil (3152)	-8.26	68.3	-4.57	232.3
Dermatan sulphate (32756)	-11.43	34.5	-7.68	174.43
Manasamine- A (12020457)	5.36	242.3	-6.09	198.4
Graciline (15384996)	-1.13	857.3	-4.15	249.4
Graciline 73162010	-13.46	54.5	-5.13	276.4
Fucoidan (92023653)	-6.44	354.4	-6.24	163.7
Macrolecrin-A 73162010	-8.37	64.6	-839	119.8

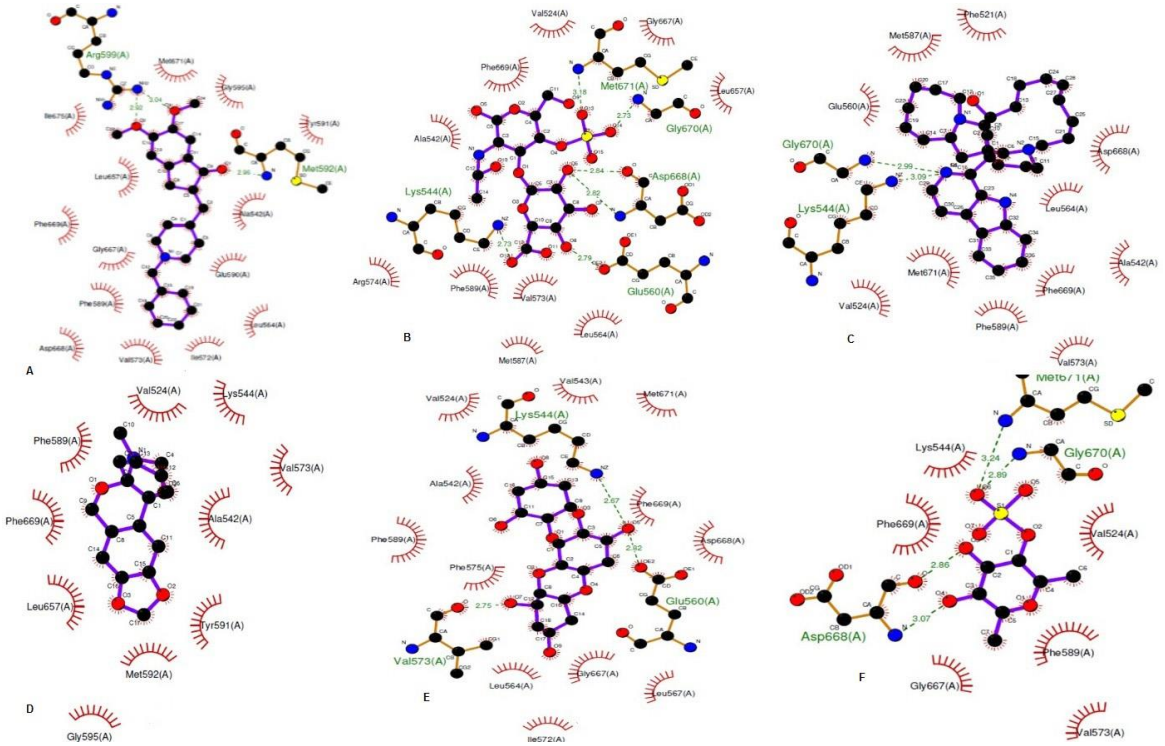

Figure 2. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with Aβ.

to other test compounds (Table 11).

4.2.3. Kinetic study of ChE and MAO in PC12 cells

The inhibitory concentration (IC_{50}) values of the tested compounds and their selectivity indexes for cholinesterase (AChE and BuChE) and MAO are summarized in Table 12. Fucoidan showed potential effects and selective inhibition for MAOA; the other test compounds showed lower potential when compared to reference compounds such as lazabemine and iproniazide. Graphical data of the Line weaver–Burk reciprocal plots are given in Figure 10A. The overlaid reciprocal Line weaver–Burk plots

Table 4. Docking score and inhibition constant of tested compounds with TrkA and PIK3

	TrkA		PIK3	
Compounds	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**	**Docking score (Kcal/mol)**	**Estimated Inhibition Constant (Ki)**
Donepezil (3152)	-13.99	49.43	-9.68	142.42
Dermatan sulphate (32756)	-11.69	53.32	-10.91	99.34
Manasamine- A (12020457)	-12.01	50.32	-6.01	323.53
Graciline (15384996)	-9.15	143.6	-5.30	234.34
Graciline 73162010	-11.60	53.34	-11.86	143.41
Fucoidan (92023653)	-8.84	174.984	-5.93	274.5
Macrolecrin-A 73162010	13.62	34.5	10.25	100.32

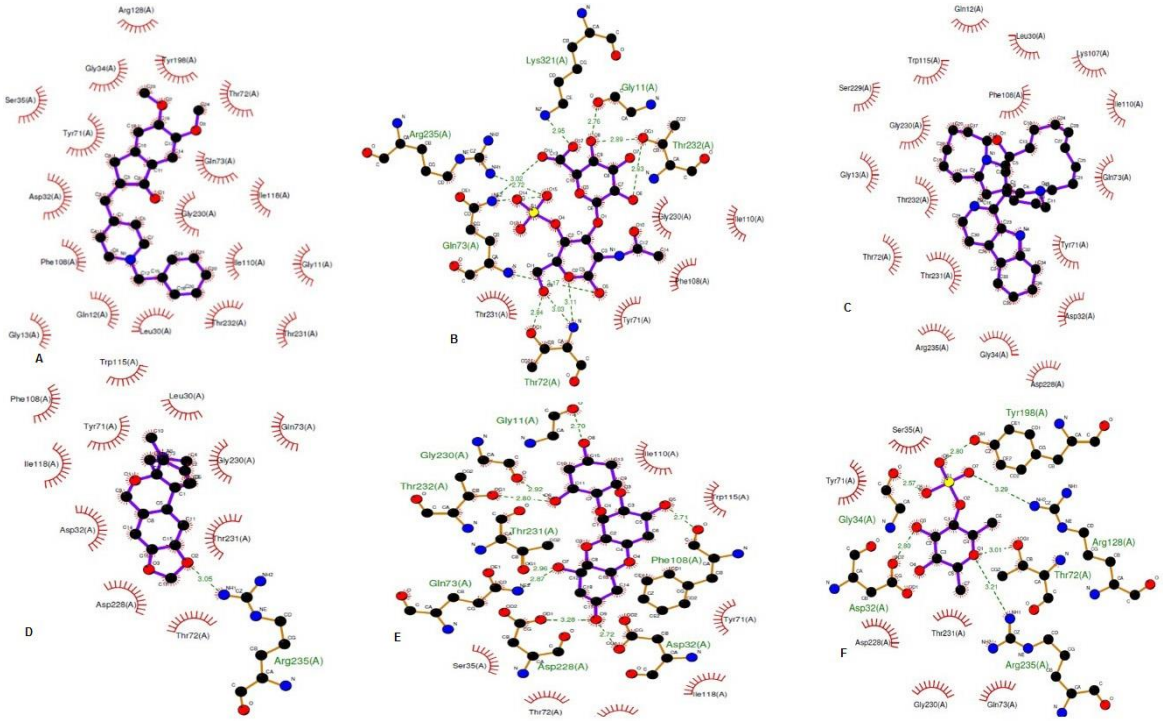

Figure 3. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with BACE-1.

(Figure 10B) showed that all plots for different concentrations of fucoidan were linear and intersected at the y-axis. Fucoidan reversed MAO-A inhibition in a time-dependent manner (Figure 10C). The AChE inhibitory activity is summarized in Table 12. There were both increased slopes and intercepts with increased inhibitory concentrations. The inhibition of AchE by fucoidan showed competitive inhibition constant (Ki) of 0.220mM. Manasamine–A exhibited potential inhibitory activity for Buch (IC_{50}=0.363mM) and fucoidan had excellent potential for inhibitory activity against AChE and MAO-A with IC_{50} values of 0.64 and 2.15 mM respectively. These results highlight that fucoidan exhibited selective MAOA and AChE inhibition activities. PC12 cells were exposed to different concentrations of test

Table 5. Docking studies of glide energy, hydrogen bound interaction, distance, and hydrophobic interaction of AchE with tested compounds

AchE				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-51.95		3.4 2.7	GLU 292, LEU 289 SER 293,ARG 296, VAL 294, GLY 122, ALA 204, GLY 121, GLY 120, GLU 202, ASP 74, TYR 124, PHE 338, TYR 341, TRP 286
Dermatan sulphate (32756)	-57.14	TYR 72 THR 75 ARG 296 ASP 74 SER 293 PHE 295	3.1 2.7 3.0 2.8 3.0 3.2	TYR 341, VAL 73, TRP 286, GLU 292, GLY 342, LEU 289, PHE 297, PHE 338, VAL 294
Manasamine- A (12020457)	-59.16	NIL	NIL	PHE 297, PHE 295, ARG 296, TYR 124, TYR 337, TYR 72, THR 75, LEU 76, LEU 289, ASP 74, TRP 286, HIS 287, GLY 342, SER 293, VAL 294
Graciline (15384996)	-40.60	ARG 296 PHE 295 GLY122	3.2 3.2 3.1	TYR 341, TYR 72, TYR 337, TRP 286, GLY 342, SER 293, SER 208, ASP 74, VAL 294, GLY 121, GLY 122, HIS 447
Graciline 73162010	-60.17	PHE 295 SER 293 HIS 287 ARG 296	3.1 3.2 2.9 2.3	TRP 286, TYR 72, TYR 341, TYR 337, TYR 124, LEU 76, LEU 289, PHE 338, PHE 297, VAL 294, GLN 291, ARG 296
Fucoidan (92023653)	-44.24	ASP 74 HIS 447	3.1 3.2	TYR 72, TYR 341, VAL 294, PHE 297, PHE 295, PHE 338, GLY 121, GLY 122
Macrolecrin-A 73162010	-61.93	ASP 74 THR 75 LEU 76 PHE 295 ARG 296	2.7 3.0 2.4 3.2 3.0	TYR 337, TYR 124, LEU 76, LEU 289, PHE

compound (20 to 80µg/ml) for 24 hr and IC_{50} values were calculated. Fucoidan at 100µg/ ml did not show significant effect on cell viability when compared to other test compounds (Figure 10D).

4.2.4. Neurite length analysis

A significant increase in percentage of neurite length was observed in fucoidan supplemented cells when compared to that of control PC12 cells that did not receive fucoidan supplementation (Figure 11 A-F). A 5 µg of fucoidan enhanced percentage of neurite length significantly when compared to that of control cells at day 7 while a similar significant (p<0.05) when the cells were subjected to 10 µg of fucoidan cells were supplemented with 20 µg of fucoidan a significant increase (p<0.05) was seen in neurite outgrowth when compared to those of control cells on 1 day. On exposure to 50 µg, 75 µg, 100 µg fucoidan, the percentage of neurite length was significantly increased (p<0.05) when compared to that of control PC12 cells that of 20 µg fucoidan supplementation on 7^{th} day. DMSO treated cells did not show significance variation from their control cells even after 7 days.

4.3. *In vitro* studies

4.3.1. Mortality rate

Mortality rate was observed in control and experimental groups of flies (Figure 12A). Mortality rate was significantly increased (p<0.05) in monocrotophos fed flies when compared to those of control flies. Monocrotophos plus fucoidan fed group showed significantly reduced mortality rate (p<0.05) when compared to monocrotophos alone fed flies. Fucoidan (VI) and donepezil (IV) fed flies did not

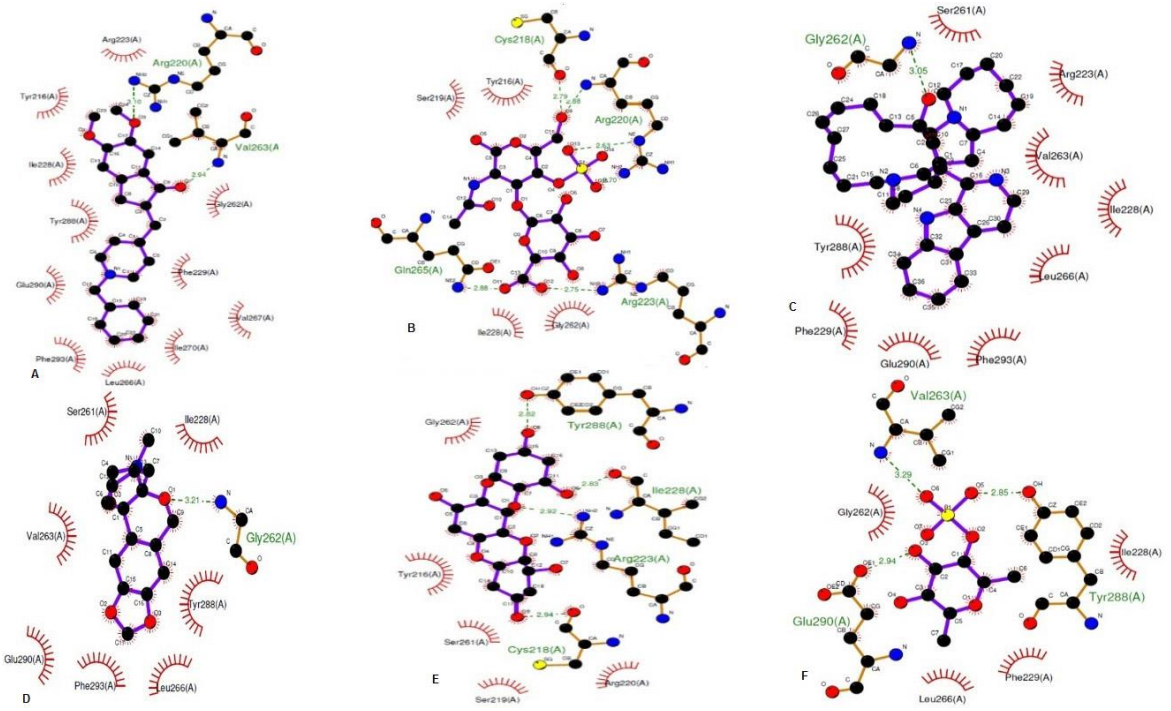

Figure 4. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with Tau protein.

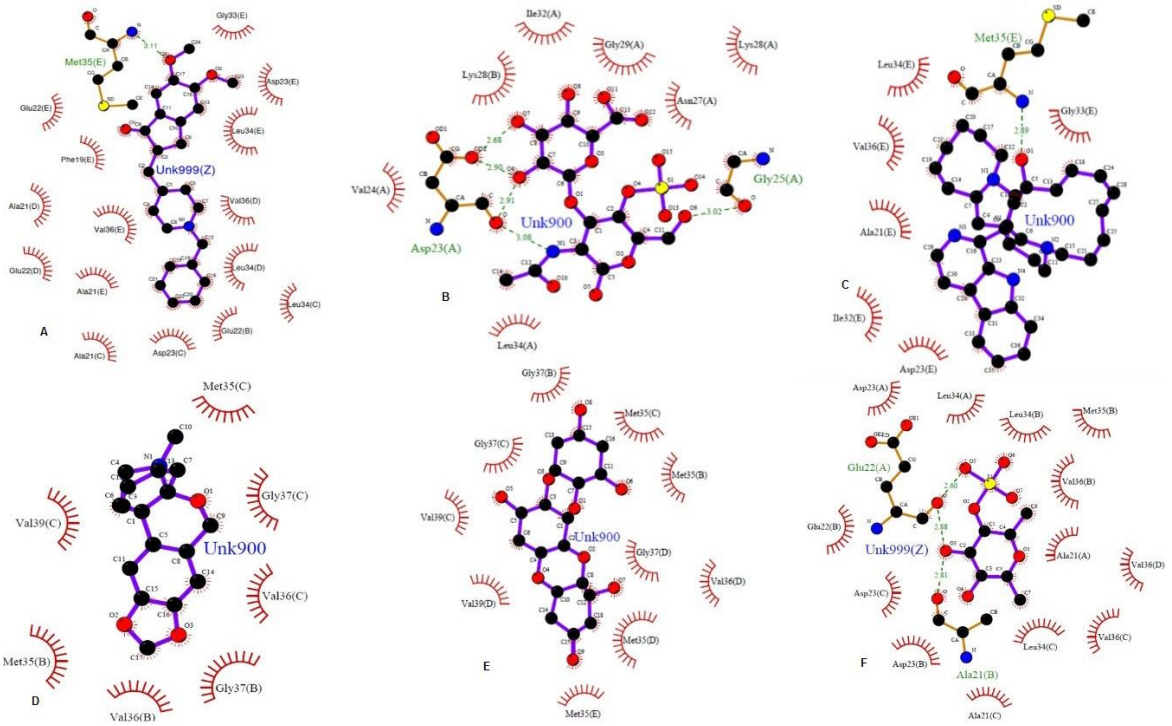

Figure 5. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with TrkA.

Table 6. Docking studies of glide energy, hydrogen bond interaction, distance, and hydrophobic interaction of β-APP with tested compounds

β-APP				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-53.89	NIL	NIL	ALA E42, LEU C17, VAL C40, VAL D40, PHE C19, PHE B19, VAL B18, LEU A17, LEU B17, ALA C42, ALA D42, LEU E17,, LEUD17, VAL E40.
Dermatan sulphate (32756)	-27.64	ASP 23 GLY 25 LYS 28	3.1 3.0 3.5	LEU B34, LEU A34, LEU C34, ILE B32, A32, GLY A33, A37, VAL A36, C36, ALA B21, A21, PHE A20, ASP A23, B23
Manasamine- A (12020457)	-19.88	MET 35	3.0	LEU- D17, C17, A17 VAL- C40,D40,E40,B18 PHE B19,C19 ALA-C42,B42
Graciline (15384996)	-9.97	NIL	NIL	VAL-E40,D40 LEU D17,A17,B17 ALA-C42,D42
Graciline 73162010	-20.07	MET 35	2.7	ALA-E42,C42,D42 VAL-C40,E40,D40 LEU-D17,C17,B17
Fucoidan (92023653)	-39.37	ALA 21 GLU 22	2.7 2.6	ASP- A23,B23 VAL-C36, B36,A36 GLU A22 ALA-A21,B21 PHE A20 ILE B32,A32 LEU-B34,A34 GLY- A33,A37
Macrolecrin-A 73162010	-41.06	MET 35 GLU 22	2.7 3.1	A17 VAL- C40,D40,E40,B18 PHE B19,C19

show any significant variation when compared to those of control flies.

4.3.2. Food intake

Food intake was observed in control and experimental groups of flies (Figure 12B). Food intake was significantly reduced (p<0.05) in monocrotophos fed flies when compared to those of control flies. Monocrotophos plus fucoidan fed group showed significantly increased food intake (p<0.05) when compared to monocrotophos alone fed flies. Fucoidan (VI) and donepezil (IV) fed flies did not show any significant variation when compared to those of control flies.

4.3.3. Level of ROS in brain

The levels of ROS in the brains of control and experimental groups were assessed (Figure 12C). Monocrotophos fed flies showed significant increased (p<0.05) in the levels of these enzymes when compared to those of control flies; monocrotophos plus fucoidan fed flies significantly decreased (p<0.05) these levels to near normal when compared to monocrotophos fed flies.

4.3.4. Cholinergic esterase activities

The activities of AchE and BuchE were assessed in the brain of control and experimental groups (Figure 12 D). The activity of AchE and BuchE were significantly reduced (p<0.05) in monocrotophos fed flies when compared with those of control. Monocrotophos plus fucoidan fed groups showed significant increase (p<0.05) in the activities of AchE and BuchE when compared to monocrotophos fed groups. Donepezil (IV) and fucoidan fed flies did not show any significant

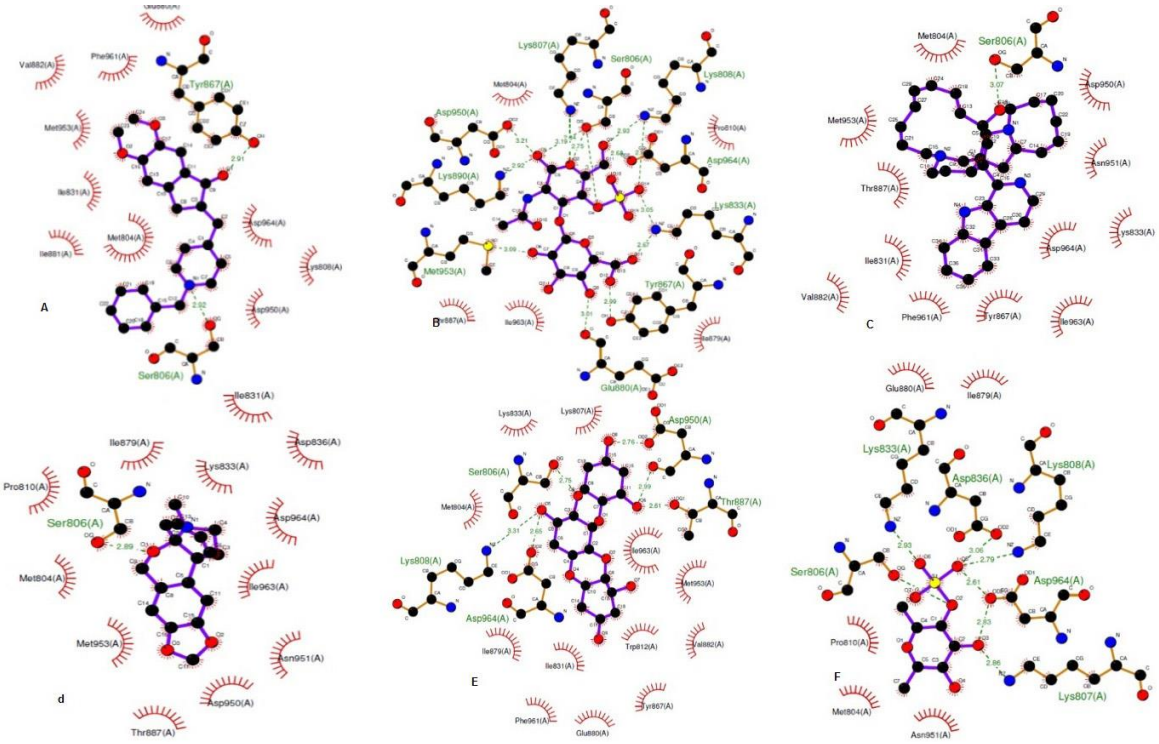

Figure 6. (A) Donepezil , (B) dermatansulfate (C) manasamine-A , (D) graciline , (E) galantamine and (F) fucoidan interaction with PI3K.

variation when compared to those of control flies.

4.3.5. Levels of proteins in brain

The protein levels were analyzed in the brain of control and experimental groups (Figure 13A and E). The levels of Tau, PIK3, TrkA, AchE, APP and BAC1 were significantly increased (p<0.05) in the monocrotophos fed group when compared with those of control. The levels of these proteins were significantly reduced (p<0.05) on monocrotophos plus fucoidan fed flies when compared to monocrotophos fed flies. Donepezil and fucoidan fed flies did not show significant variation in the levels of proteins when compared to those of control flies.

4.3.6. The levels of neurotransmitters in brain

The levels of dopamine, glutamate, GABA, octopamine, serotonin and tryptoamine were assessed in the brains of control and experimental groups (Figure 13B and D). The levels of these neurotransmitters in the monocrotophos fed flies were significantly reduced (p<0.05) when compared with those of control. However, the levels showed a comparative increase in monocrotophos plus fucoidan fed groups highlighting the effect of fucoidan on maintaining neurotransmission. In fucoidan (VI) and donepezil (IV) fed flies, there was no significant variation when compared to those of control brain.

4.3.7. Oxidative stress induced molecular analysis

The levels of SOD, CAT, GSH and LPO were assessed in the brains of control and experimental groups (Figure 14A and B). Monocrotophos fed flies showed significant changes (p<0.05) in the levels of these enzymes when compared to those of control flies; monocrotophos plus fucoidan fed flies significantly altered (p<0.05) these levels to near normal when compared to monocrotophos fed flies.

4.3.8. Neuroinflammation molecular analysis

The levels of TNF-α and IL-6were assessed in the brains of control and experimental

Table 7. Docking studies of glide energy, hydrogen bond interaction, distance, and hydrophobic interaction of BACE1 with tested compounds

BACE1				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-62.40	GLN 73 THR 72	3.4 2.7	THR 232,GLY11, THR 231, GLY 13, ILE 110, PHE 108, ASP 32, SER 35, TYR 71, ASP 228, GLY 34, TYR 198,ARG 128, ILE 126, TRP 115, THR 329, ILE 118, ARG 235, SER 229, GLY 230, TRP 115, LEU 30, GLN 12,ILE 110.
Dermatan sulphate (32756)	-78.79	GLY 11 ARG 235 GLN 73 THR 232 LYS 321	2.8 2.7 3.2 3.0 2.9	PHE 108, GLN 12, GLY 74, TYR 71, GLY 230, ASP 228, THR 231, VAL 332, ASN 233, LYS 321
Manasamine- A (12020457)	-64.42	GLY230	2.8	ILE 110, ARG 235, GLN 12 LYS 107, GLY 13, ILE 226, THR 72, VAL 332, TYR 198 SER 35, ASP 228, GLY 34 ASP 32, TYR 71, ASN 233 THR 231, THR 232, GLY 230 GLN 73, GLY 11, GLY 74 SER 229. ILE 118, TYR 14 TRP 115, LEU 30, PHE 108
Graciline (15384996)	-38.97	ARG235	3.1	ILE 110, GLN 12, LYS 107, GLY 13, ILE 226, THR 72, VAL 332, TYR 198 SER 35, ASP 228, GLY 34 ASP 32, TYR 71, ASN 233 THR 231, THR 232, GLY 230 GLN 73, GLY 11, GLY 74 SER 229. ILE 118, TYR 14 TRP 115, LEU 30, PHE 108
Graciline 73162010	-69.59	GLY 230 THR 232 GLY 11 GLN 73 ASP 32	2.9 3.1 2.7 2.2 2.7	ILE 110, GLN 12, GLY 13 GLY 74, TRP 115, PHE 108 ILE 118, LEU 30, TYR 71 GLY 34, SER 35, ASP 228 THR 72.
Fucoidan (92023653)	-52.06	ARG 235 THR 72 TYR 198 GLY 34 ARG 128 ASP 32 GLY 230	3.0 2.8 2.7 3.1 2.0	TYR 71, GLN 73, THR 231, VAL 332, ILE 226 ASP 228, SER 35, PRO 70, ILE 118
Macrolecrin-A 73162010	-64.28	GLN 12 PHE 108	2.9 2.8	GLY 34, TYR 198,ARG 128, ILE 126, TRP 115, THR 329, ILE 118, ARG 235, SER 229, GLY 230, TRP 115, LEU 30

groups (Figure 14C). Monocrotophos fed flies showed significantly reduced levels (p<0.05) of these proteins when compared to those of control flies; in the monocrotophos plus fucoidan group there was an increased level (p<0.05) of TNF-α and IL-6 when compared to those of monocrotophos fed flies.

4.3.9. Apopototic regulation molecular analysis

The levels of Bcl2 and caspase-3 proteins were assessed in the brains of control and experimental groups (Figure 14D). Monocrotophos fed flies showed significantly reduced levels (p<0.05) of these proteins when compared to those control

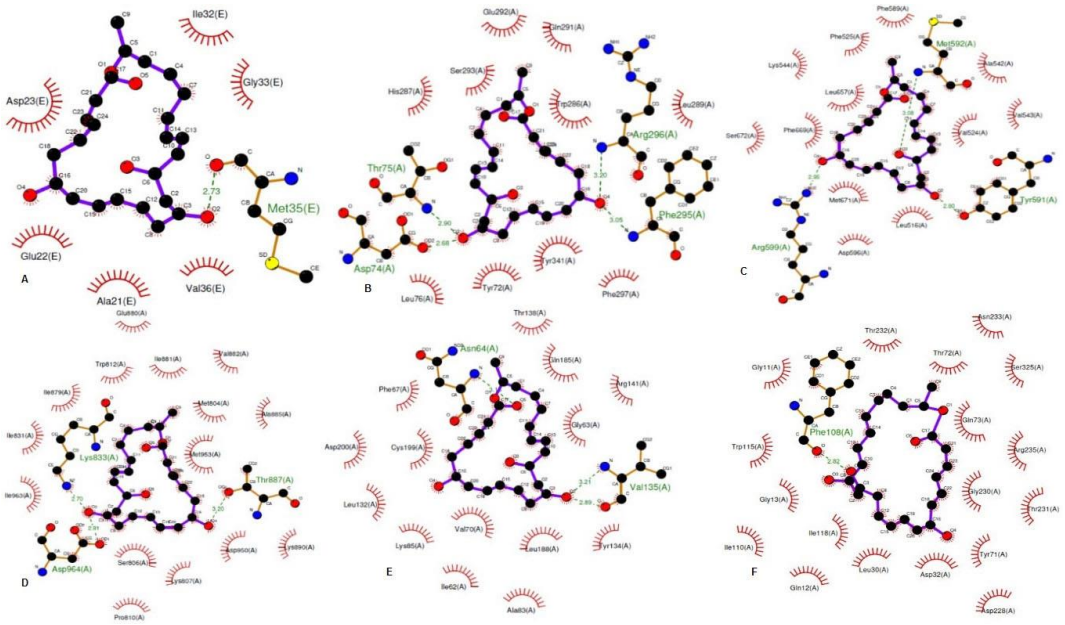

Figure 7. Macrolactin –A interaction with (A) Amyloid-beta protein , (B) AchE , (C) TrkA , (D) PI3K, (E) Tau /GSK-3β and (F) BACE-1.

Table 8. Docking studies of glide energy, hydrogen bond interaction, distance, and hydrophobic interaction of Tau/3βGSK with tested compounds

Tau/3βGSK				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-39.96	ARG 220	3.1	LEU 266, PHE 293, TYR 288 GLY 230, TYR 216, SER 215 LEU 227, ARG 223, ILE 228 GLY 262, PHE 229, VAL 263 LYS 292, GLU 290, PRO 294 VAL 267, ILE 270
Dermatan sulphate (32756)	-42.13	CYS 218 ARG 220 GLN 265 ARG 223	2.8 3.0 2.8 3.1	SER 219, TYR 216, ILE 228, SER 261, GLY 262
Manasamine- A (12020457)	-47.10	GLY262	2.1	VAL 263, SER 261, TYR 216, ARG 223, GLN 265, ASP 260, PHE 229, ILE 228, LEU 266, GLU 290, PHE 293, TYR 288.
Graciline (15384996)	-28.53	GLY262	3.0	SER 261, VAL 263, GLU 290, TYR 288, PHE 293, LEU 266, PHE 229,ILE 228.
Graciline 73162010	-41.95	CYS 218 ARG 223 ILE 228 TYR 288	2.1 2.3 3.0 2.7	TYR 216, SER 219, ARG 220 GLN 265, SER 261, ASP 260 LEU 227, GLY 262, PHE 229 VAL 263
Fucoidan (92023653)	-33.31	TYR 288 VAL 263 GLU 290	3.4 3.0 2.9	 ILE 228, PHE 229, LEU 266 PHE 293, VAL 267, ILE 270, LYS 292, GLY 262 , SER 261.
Macrolecrin-A 73162010	-56.45	ASN 64 ASP 200 VAL 135	3.2 3.4 2.9	LYS 292, GLU 290, PRO 294

Table 9. Docking studies of glide energy, hydrogen bond interaction, distance, and hydrophobic interaction of TrkA with tested compounds

TrkA				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-56.68	ARG 599 MET 592	3.2 2.8	VAL 573, ALA 542, TYR 591 HIS 594, LEU 516, GLY 595 MET 671, ILE 675, ASP 596 SER 672, TYR 676, LEU 657 VAL 524, PHE 669, PHE 589, ASP 668, GLY 667, ILE 572 LEU 567, PHE 575, LEU 564 GLU 590
Dermatan sulphate (32756)	-60.21	GLU 590 ASP 668 GLU 560 LYS 544 GLY 670 MET 671	2.5 3.0 3.3 2.4 2.2 3.0	TYR 591, ALA 542, MET 592, PHE 589, VAL 573, LEU 657, GLY 667, ARG 574, PHE 575, LEU 564, MET 587, VAL 524, PHE 669
Manasamine- A (12020457)	-75.96	GLY670	3.4	MET 587, GLY 670, ARG 673, LEU 546, GLY 522, ASP 556, GLU 560, MET 671,GLY 519, PHE 669, VAL 524, LEU 657 , GLU 590, ALA 542, VAL 573, PHE 589, LEU 563, LEU 564, LEU 567, PHE 646, ASP 668, LYS 544, PHE 557, PHE 521
Graciline (15384996)	-36.43	MET592	2.1	ASP 596, LEU 516, MET 671 PHE 669, GLY 667, GLY 670, PHE 589, ASP 668, LYS 544 VAL 524, GLU 590, VAL 543 VAL 573, ALA 542, LEU 657
Graciline 73162010	-57.37	GLU560	3.2	LYS 544, GLY 670, ASP 668, ILE 666, GLY 667, ILE 572, LEU 567, LEU 564, PHE 575, VAL 573, ARG 574, PHE 669, LEU 657, GLU 590, PHE 589, ALA 542, PHE 525, VAL 524, VAL 543, MET 671
Fucoidan (92023653)	-40.51	MET671 ASP668	3.2 2.0	TYR 72, TYR 341, VAL 294, PHE 297, PHE 295, PHE 338, GLY 121, GLY 122.
Macrolecrin-A 73162010	-58.65	MET592 TRY591 ARG599 SER673	2.8 3.1 2.7 3.0 3.0	TYR 337, TYR 124, LEU 76, LEU 289, PHE

flies; in the monocrotophos plus fucoidan group there was an increased level (p<0.05) of Bcl2 and casp-3 when compared to those of monocrotophos fed flies.

4.3.10. *mRNA* levels in brain

m-RNAs levels were analyzed in the brain of control and experimental groups (Figure 15A, B , C and D). The *mRNA* levels of *Tau, PKI3,TrkA AchE, β-APP* and *BACE1,* were significantly increased in monocrotophos fed group when compared with those of control. Monocrotophos plus fucoidan fed flies showed significant decrease (p<0.05) in the *m-RNAs* levels when compared to monocrotophos fed flies. Donepezil and fucoidan fed flies did not show any significant (p>0.05) variation in the level of *m- RNA* when compared to those of control flies.

Also *m-RNAs* levels were analyzed in the brain of control and experimental groups (Figure 16). The *mRNA* levels of *mTOR, VMAT* and *SERT* were significantly increased in the monocrotophos fed group when compared with those of control. Monocrotophos plus fucoidan fed flies showed significant decrease (p<0.05) in the *m-RNAs* levels when compared to monocrotophos fed flies.

Table 10. Docking studies of glide energy, hydrogen bond interaction, distance, and hydrophobic interaction of PI3K with tested compounds

PI3K				
Compounds	**Glide energy (Kcal/mol)**	**Hydrogen Bond interaction**	**Distance (Å)**	**Hydrophobic Interaction**
Donepezil (3152)	-55.48	VAL 882 TYR 867 SER 806	3.2 2.8 2.3	VAL 573, ALA 542, TYR 591 HIS 594, LEU 516, GLY 595 MET 671, ILE 675, ASP 596 SER 672, TYR 676, LEU 657 VAL 524, PHE 669, PHE 589, ASP 668, GLY 667, ILE 572 LEU 567, PHE 575, LEU 564 GLU 590
Dermatan sulphate (32756)	-61.90	LYS 890 SER 806 LYS 807 LYS 808 LYS 833 ASP 964 GLU 880	2.4 3.2 3.0 3.2 3.0 2.4 2.5	THR 886, ASP 950, ILE 963 ASN 951, ASP 836, LYS 809 PRO 810, ILE 831, ILE 879 TYR 867, ILE 881, TRP 812 MET 804, PHE 961, VAL 882 MET 953, THR 887
Manasamine-A (12020457)	-59.80	SER 806	3.0 3.0 2.0 2.1 3.1	ALA 885, ILE 881, VAL 882, PHE 961, GLU 880, ILE 879, ILE 831, ALA 805, ILE 963, LYS 833, ASP 964, LYS 807, LYS 808, MET 804, ASN 951, ASP 950, PRO 810, MET 953, TRP 812, THR 887, THR 886, LYS 890.
Graciline (15384996)	-37.38	THR 887 ASP 950 ASP 964 SER 806		ILE 963, ALA 885, MET 953, LYS 890, ASN 951, LYS 807, LYS 808, PRO 810, MET 804 , LYS 833, ILE 831, ILE 879, PHE 961, TYR 867, ILE 881, VAL 882, GLU 880, TRP 812.
Graciline 73162010	-64.59	THR 887 ASP 950 ASP 964 SER 806	32.1 3.2 3.3 2.1	ILE 963, ALA 885, MET 953 LYS 890, ASN 951, LYS 807, LYS 808, PRO 810, MET 804, LYS 833, ILE 831, ILE 879, PHE 961, TYR 867, ILE 881 VAL 882, GLU 880, TRP 812.
Fucoidan (92023653)	-44.70	ASP 950 LYS 807 ASN 951 ASP 964 LYS 808 LYS 833	3.1 3.0 2.8 2.9 3.0 2.0	PRO 810, SER 806, ASP 836, ILE 831, ILE 963, MET 804, ILE 879, THR 887
Macrolecrin-A 73162010	-55.48	ASP 950 ASP 954 LYS 833	3.0 2.8 2.7	ASP 950, PRO 810, MET 953 TRP 812, THR 887, THR 886

Donepezil and fucoidan fed flies did not show any significant variation in the level of *m- RNAs* when compared to those of control flies.

4.3.11. T and *Y Maze*

The latency of learning memory and aggressivity were assessed in *D. melanogaster* in control and experimental groups (Figure 17 A). There was significantly delayed acquisition latency (p<0.05) in monocrotophos fed flies when compared to those of control flies, indicating memory deficits. Monocrotophos plus fucoidan treated group significantly improved acquisition latency (p<0.05) when compared to those of control flies, indicating improved learning and memory. Fucoidan (VI) and donepezil (IV) fed flies did not show any significant variation when compared to control flies.

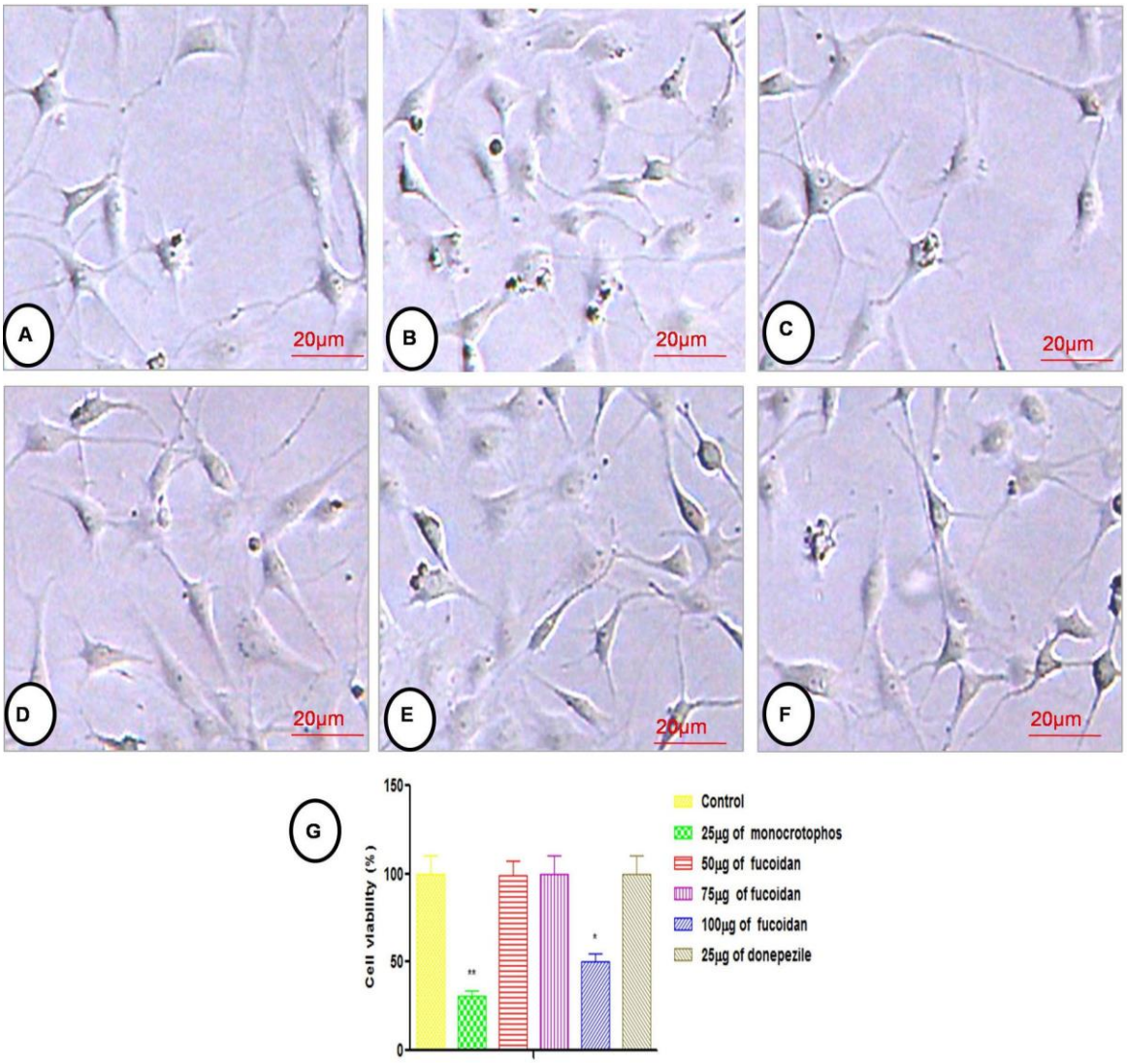

Figure 8. The neuroprotective effects of fucoidan in PC12 cells. Cells were treated with different concentrations of fucoidan and then incubated with or without monochrotophos for 7 days (n=3).

4.3.12. Crawling behaviour

The latency time of locomotor behavior was assessed in *D. melanogaster* in control and experimental groups (Figure 17B). The latency time of *D. melanogaster* was significantly reduced (p<0.05) in monocrotophos fed flies when compared to those of control flies. Monocrotophos plus fucoidan fed group showed significant increase (p<0.05) in the latency time when compared to monocrotophos fed alone flies. The fucoidan (VI) and donepezil (IV) fed flies did not show any significant variation when compared to control flies.

4.3.13. Olfactory and courtship index

The index of olfactory and courtships behavior was assessed in *D. melanogaster* in control and experimental groups (Figure 17C and D). The olfactory and courtships of *D. melanogaster* was significantly reduced (p<0.05) in monocrotophos fed flies when compared to those of control flies. Monocrotophos plus fucoidan

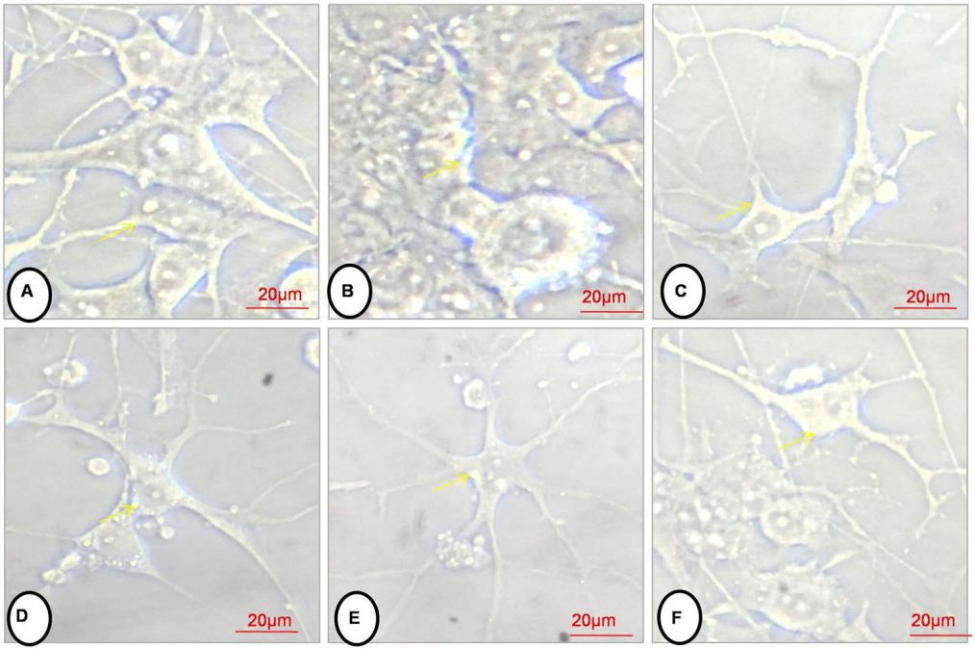

Figure 9. Effects of fucoidan on neuritent growth of neurons in PC12 cells Cells were treated with 6.25-12.5µg concentration of fucoidan (C-E) and then incubated with or without monocrotophosfor 7 days (n=3).

Table 11. Permeability results (P_e x 10^{-6} cm s^{-1}) from the PAMPA-BBB assay for selected test compounds and commercial drugs, with their predicted penetration into the CNS

Tested compounds	**Permeability (Pe x 10^{-6} cm s^{-1})[1]**	**Prediction**
Manasamine A	8.81±1.4	CNS+
Fucoidan	10.4±0.9	CNS+++
Graciline	6.57±0.7	CNS+
Dermatan sulphate	0.0±0.0	NIL
Macrolecrin-A	0.0±0.0	NIL
Testosterone	15.6±1.03	CNS+
Verapamil	13.2±0.85	CNS++
β-Estradiol	8.90±0.64	CNS+
Progesterone	6.62±0.43	CNS+
Clonidine	4.74±0.26	CNS++
Corticosterone	4.92±0.42	CNS+
Piroxicam	1.91±0.17	CNS+
Hydrocortisone	1.57±0.12	CNS++
Dopamine	0.15±0.01	CNS+
Data are the mean± SEM of three independent experiments.		

fed group showed significant increase (p<0.05) in the index time when compared to monocrotophos fed alone flies. The fucoidan (VI) and donepezil (IV) fed flies did not show any significant variation

Table 12. Cholinesterases and human recombinant MAO isoforms inhibitory activities of tested compounds and reference compound

Tested compounds	IC_{50} µM		Selective Index (SI)	IC_{50} µM		Selective Index (SI)
	AchE	Buch	AchE/Buch	MAO-A	MAO-B	MAOA/MAO-B
Dermatansulphate	7.1±0.5	3.0±0.1	7.1±0.5	7.1±0.5	7.4±0.5	10.1±1.5
Manasamine- A	50.1±4.7	42.4±3.9	42.4±3.9	22.1±1.9	30.2±3.0	11.4±1.0
Graciline	24.6±1.4	17.9±1.0	42.4±3.9	32.0±1.3	72.4±2.9	22.1±1.4
Fucoidan	75.2±4.3	70.1±3.0	42.4±3.9	12.3±1.5	52.0±3.1	36.4±1.7
Macrolectin-A	2.8±0.75	2.3±0.4	42.4±3.9	65.1±4.2	25.1±3.7	52.5±3.2
Donepezile	60.7±2.7	61.4±2.2	42.4±3.9	42.6±3.7	40.4±1.9	43.1±3.4
Galatamine	17.3±1.6	12.9±1.0	42.4±3.9	54.4±3.9	61.2±3.2	52.0±1.2
Lazabemide	nt[c]	nt[c]	42.4±3.9	72.4±3.7	32.4±1.1	22.4±1.9
Iproniazid	56.2±2.8	51.0±2.1	42.4±3.9	62.4±4.0	42.4±3.9	41.4±2.5

[a]IC_{50}: 50% inhibitory concentration (means ±SEM of three experiments). [b]Inactive at 100mM (highest concentration tested), at higher concentrations the compounds precipitate. [c]nt=not tested.

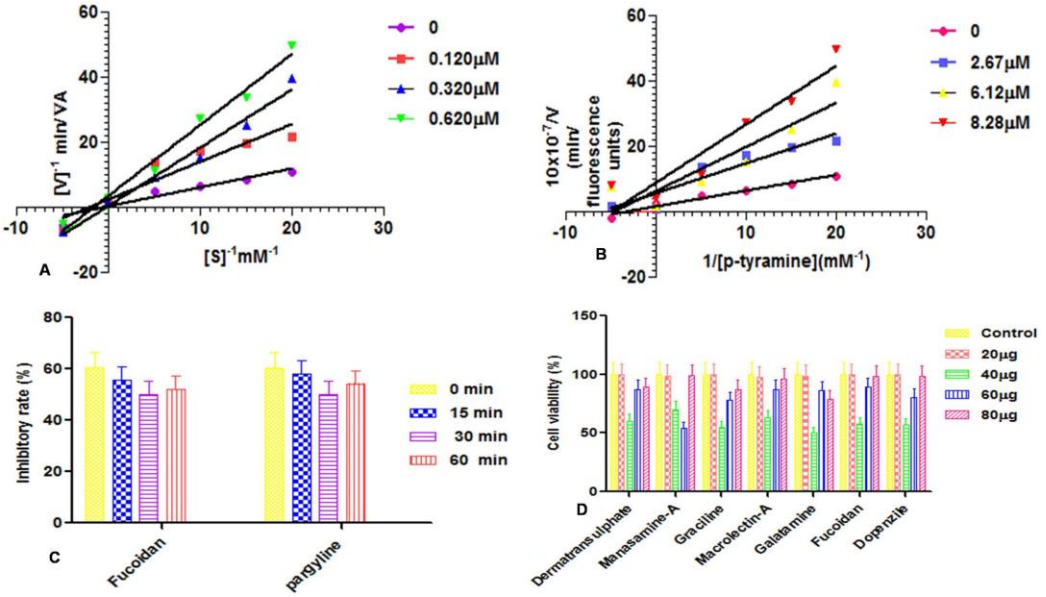

Figure 10. Kinetic studies on the mechanism of (A) AChE and (B) MAO-B inhibition by fucoidan. (C) Reversibility studies of MAO-A inhibition by fucoidan and pargyline. (D) Cell viability of PC12 cells and Cells were treated with different concentrations of fucoidan and then incubated with or without monochrotophos for 7 days (n=3). Data were expressed as percentage of cell viability. Values indicate mean ± SEM (n=3) and those having same alphabet did not deviate significantly by at $p<0.05$.

when compared to control flies.

5. DISCUSSION

5.1. *In silico studies*

Molecular docking studies were carried out against anti-Alzheimer's drug targets such as AchE, APP, BACE1, Tau/GSK-3β, TrkA and PI3K proteins for understanding their binding efficiency. Drug discovery is gaining momentum; it can help to identify novel inhibitors well before the start of pre-clinical /clinical studies in animals and humans. The currently used donepezil drug is an effective anti-AChE for AD; but it causes more side effects in patients (30). Initially twenty five drugs were selected from

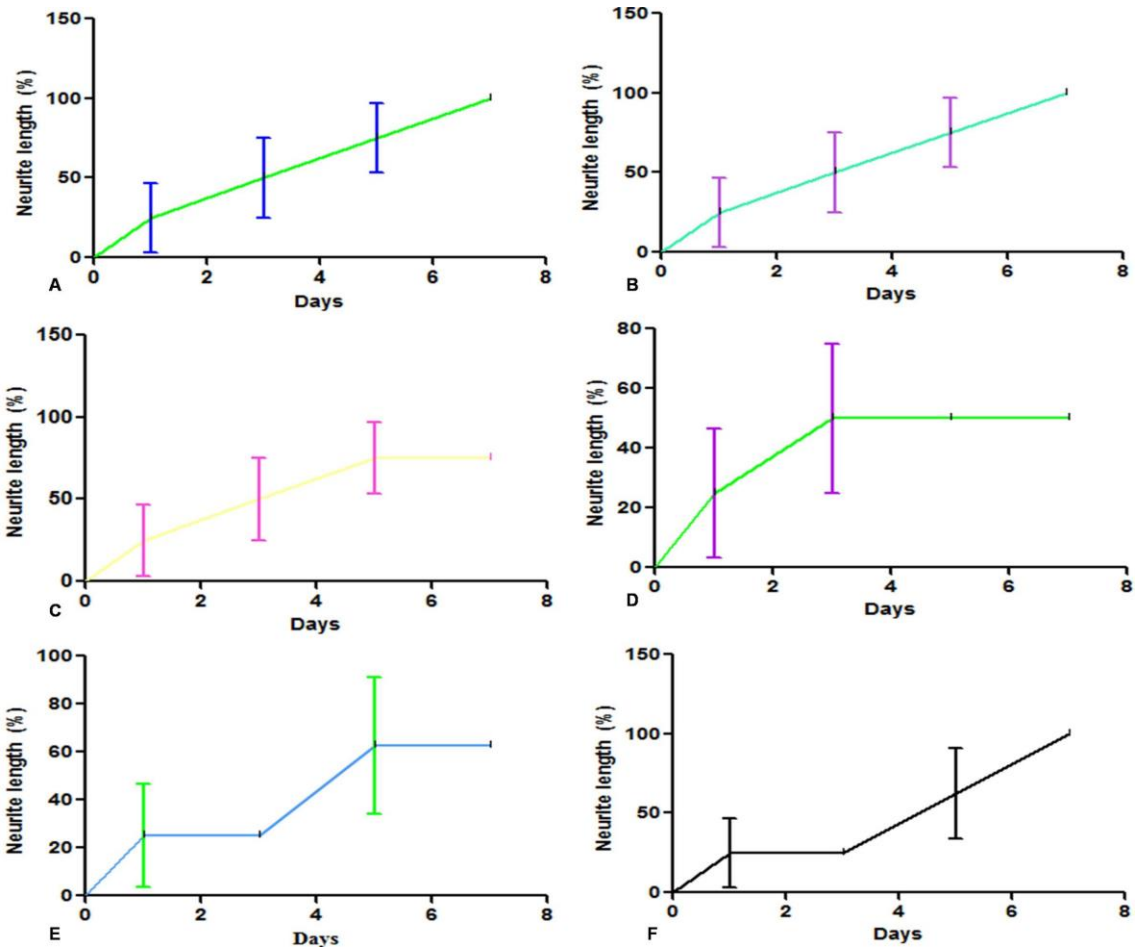

Figure 11. Morphological changes of neurite length in PC12 cell lines and cells were exposed to different concentrations (0-20µg) of fucoidan for 7 days. The percentage of neurite lengths was the highest in the experimental group supplemented with 20µg fucoidan on 7th day. Data were expressed as mean ±SEM (n=3). $p \leq 0.05$ was considered significant when compared to that of cells at 0 day (before fucoidan supplementation).

chemical data base; six test compounds were selected based on their interaction with target proteins. Fucoidan interacted with BACE1 at Asp32 and Asp228 residues as evidenced by Huang *et al.,* (31). *In silico* studies gracilins interacted with methionine 35 (Met35) (32). In our study, fucoidan interacted with Met35 regulating Aβ aggregation in neurons. Docking results revealed that fucoidan bound to active sites such as TYR 124, PHE 295, VAL 294, TYR 337, TYR 72, TRP 286, SER 293, PHE 297, PHE 338 and TYR 341 at AchE. Facoidan intraction with proteins showed high binding energy of -8.88 and -51.95 kcal/mol suggesting that fucoidan could maintain the cognitive behaviors. Gracilins regulate the β- secreatase-1 and Tau protein in neurons (32).The comparison of donepezil with the test compound suggested the potential of fucoidan to maintain the β- secreatase-1 and Tau levels in brains of flies. In our study, TrkA interacted with fucoidan residues of Asp688 and Lys544 as evidenced by Wood *et al.,* (33) suggesting the regulated synaptic transmission of brain. Docking results suggested that fucoidan exhibited binding with AD target proteins.

5.2. *In vitro* studies

The pharmacokinetics of subcutaneous low molecular weight drugs have a more predictable bioavailability and cross the BBB in the central nervous system (CNS) (34). Fucoidan has molecular

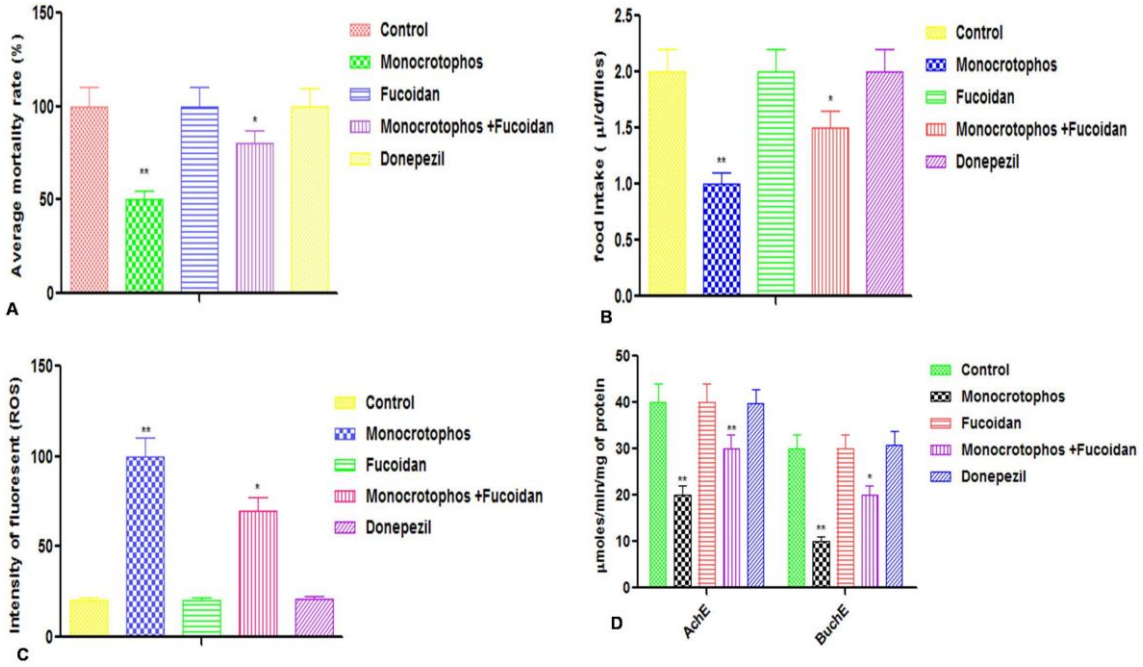

Figure 12. The effects of fucoidan on (A) mortality, (B) food intake, (C) ROS level and (D) AchE and Buch in control and experimental groups of flies. Data are expressed as mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Values not sharing same alphabets differ significantly by at $p<0.05$.

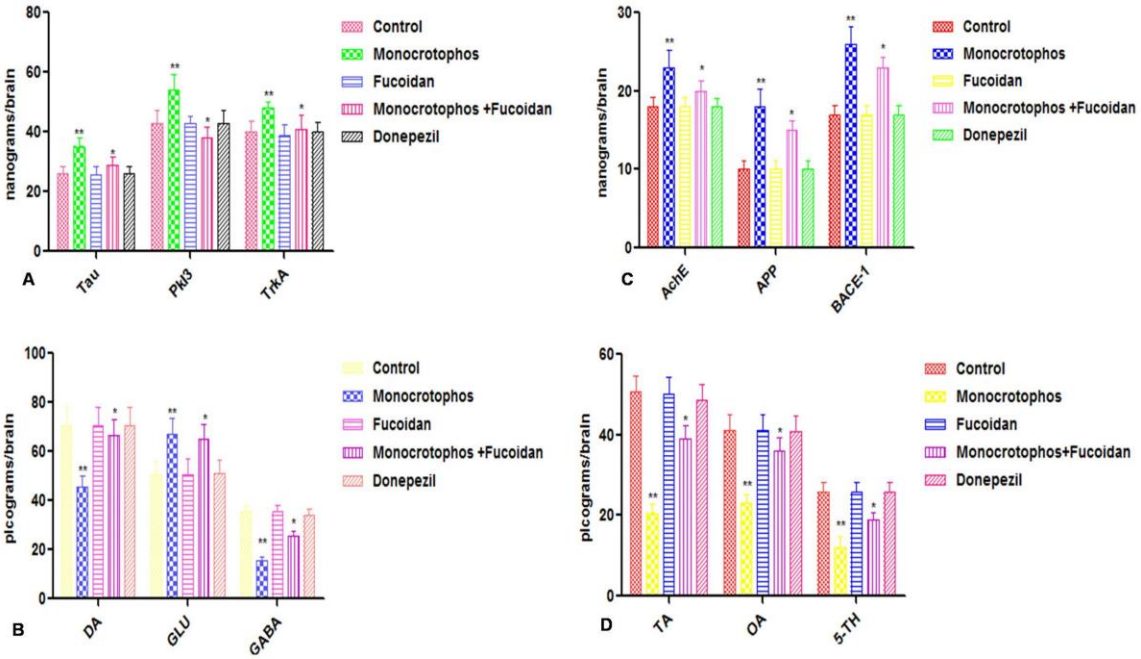

Figure 13. Effects of fucoidan on (A) AchE, APP and BACE-1, (B) DA, GABA and GL , (C) Tau, PkI3 and TrkA, (D) TA, OA and 5-TH and U levels and activities in brain of control and experimental groups of *D. melanogster.* Data are mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Values not sharing same alphabets differ significant by at $p<0.05$

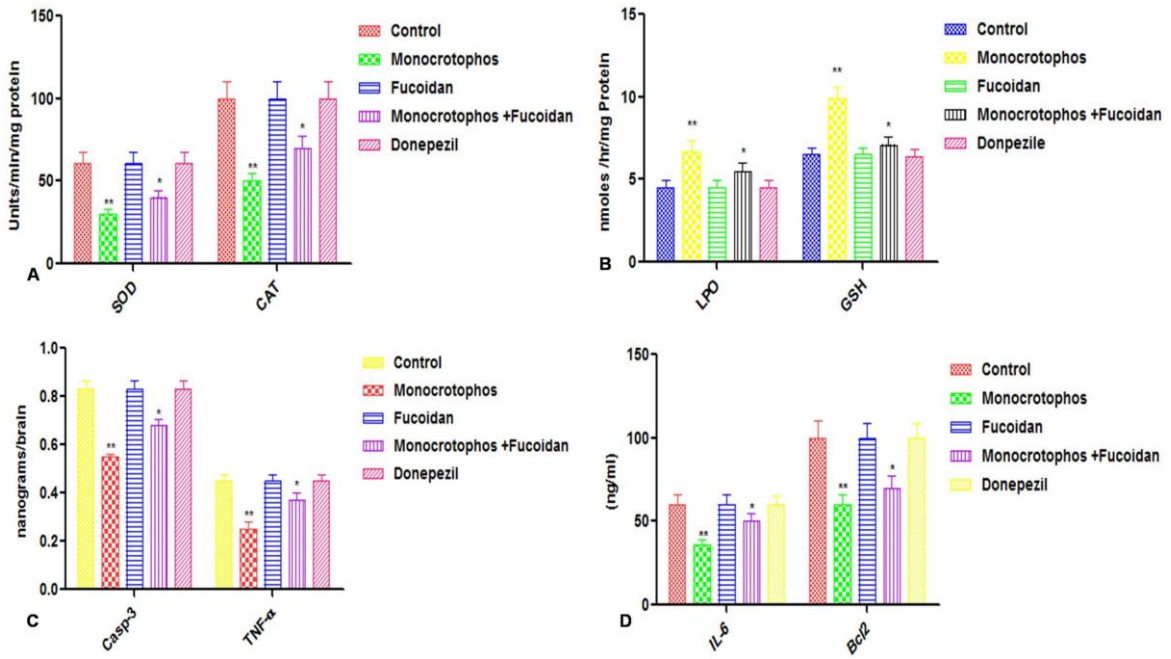

Figure 14. Effects of fucoidan on (A) SOD and CAT; (B**)** LPO and GSH , (C) TNF-α and IL-6 , (D) Bcl2 and caspase-3 levels in brain of control and experimental groups of *D. melanogster.* Data are mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Values not sharing same alphabets differ significantly by at $p<0.05$.

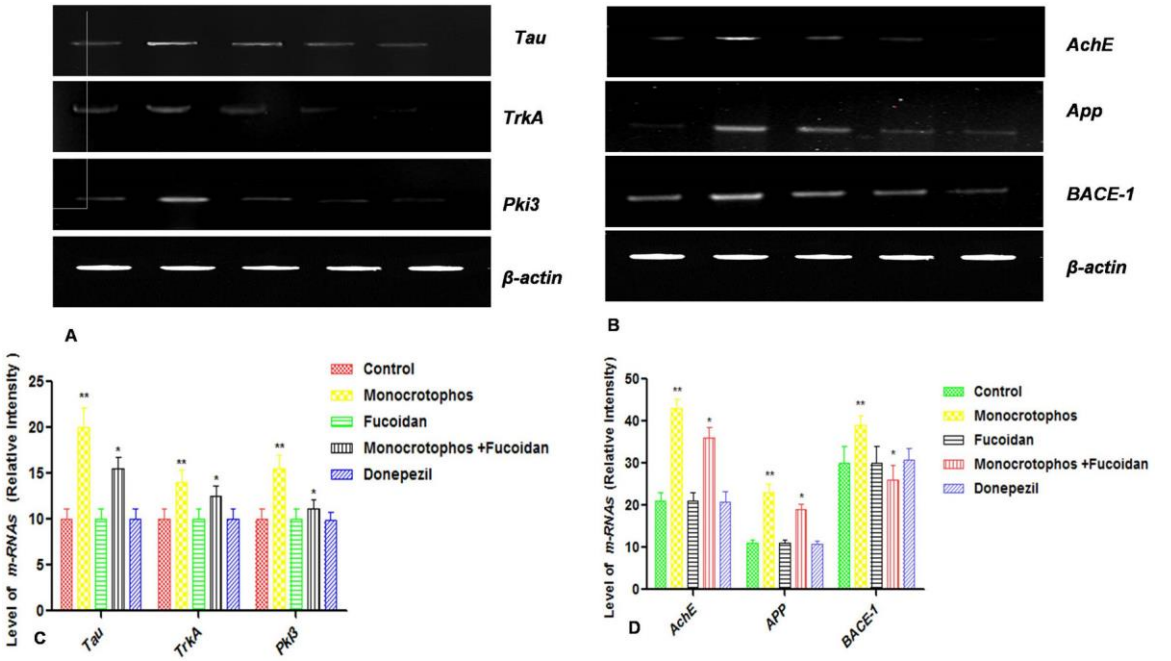

Figure 15. Effects of fucoidan on (A) *Tau, TrkA, Pkl3*, (B) *AchE, APP* and *BAEE-1* in brain of control and experimental groups of *D. melanogaster*. (C and D) quantification of *m-RNAs* by using image J software. Data are mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Values not sharing same alphabets differ significantly by at $p<0.05$.

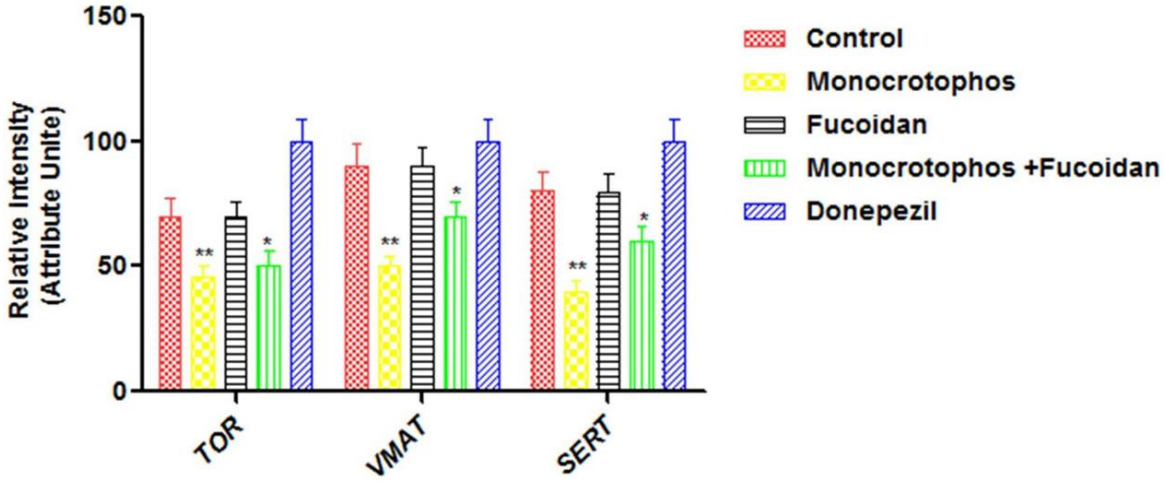

Figure 16. Effects of fucoidan on *TOR, VMAT* and *SERT* in brain of control and experimental groups of *D. melanogster*. Data are mean ± SEM (n=3) and statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Values not sharing same alphabets differ significantly by at $p<0.05$.

weight of 242.242; it easily can overcome the blood –brain barrier. Fucoidan was non-toxic to PC12 cells and might be drug candidate for treating AD. Due to the ChEs inhibitory activities of donepezil, rivastigmine and galanthamine, these are used drugs treat AD (35). Fucoidan exhibited strongest inhibition against BuChE with IC_{50} value of 0.323mM, which was 9 times more than that of donepezil (IC_{50}=2.32mM). Fucoidan showed the highest selectivity with a selectivity index of 0.0392. This suggested that the polysulfuric acids might be favorable for ChEs inhibition. Fucoidan showed potential to inhibit ChEs (BuChE: IC_{50}=1.23mM; AChE: IC_{50}=0.454mM) and MAOs (MAO-A: IC_{50}=13.4mM; MAO-B: IC_{50}=3.14mM) suggesting fucoidan to be a mixed-type of inhibitor interacting with dual sites (peripheral anionic site and catalytic active site) of AChE. Reversible inhibitors of MAO-B have important advantages more than the irreversible inhibitors in AD treatment. Therefore, to study whether fucoidan was a reversible or irreversible MAO-B inhibitor, time dependent inhibition was done with an irreversible inhibitor, pargyline, as reference compound (35). Fucoidan proved to be a reversible MAO-A inhibitor as evidenced by the time-dependent decrease and its inhibitor activities. In the current study, Line weaver–Burk plots showed that fucoidan acted as a competitive MAO-A inhibitor; this result further proved that fucoidan was reversible MAO-A inhibitor. PC12 cells were differentiated with different concentrations of fucoidan for 7 days. The morphology change of different concentrations of fucoidan on PC12 was relevant to percentage of neurite length. Further, the effect of different concentrations of fucoidan from 0 to day 7 in PC12 cells was assessed in the variations of the proteins and *mRNA* involved in neuronal differentiation, survival and synaptic plasticity and hence in pathological conditions like-Alzheimer disease .

5.3. *In vivo* studies

Decreased energy intake (food consumption) was observed in Alzheimer's disease in *Drosophila melanogaster* (36). Food consumption was significantly reduced in monocrotophos fed flies; fucoidan maintained the food intake of flies. The mortality rate was significantly increased in monocrotophos fed flies; fucoidan in the diet did not cause adverse effects of mortality rate. The accumulation of Tau is associated with AD in flies (37).Tau protein level was significantly increased in monocrotophos fed flies but fucoidan maintained the tau level in neurons of flies. *In vitro* studies using dichlorodiphenyltrichloroethane (DDT) showed the accumulation of amyloid- β precursor protein (AβPP) and β-site AβPP-cleaving enzyme-1 (BACE-1)

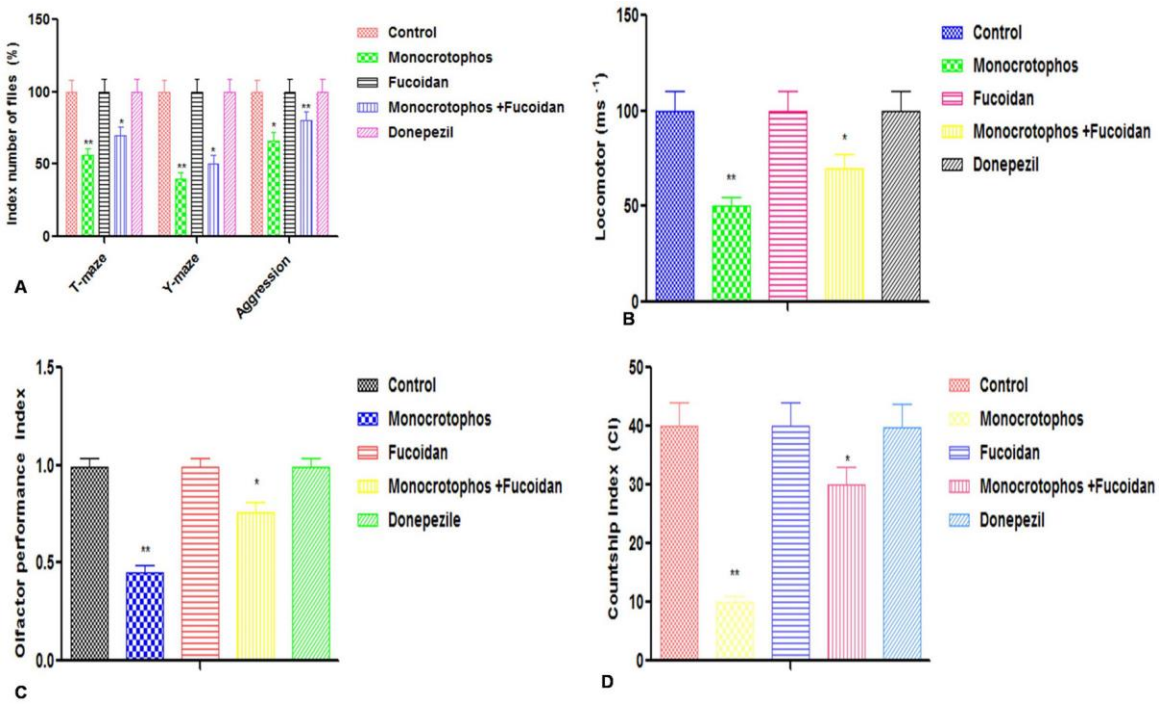

Figure 17. Effects of fucoidan on (A) learning and memory , (B) movement , (C) olfactory and (D) courtships behaviours in control and experimental groups of *D. melanogster*. Data are mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test and values having same alphabet did not deviate significantly by at $p<0.05$.

impairing the clearance and extracellular degradation of amyloid-β peptides as well as increase in memory loss and reduction in motor activity in animals (38). Our findings showed that the *mRNA* level of AβPP was significantly reduced in monocrotophos fed flies but fucoidan regulated the AβPP pathways of flies. An increase in PI3K/Akt proteins was found in the postmortem AD in patients (39).The *mRNA* and protein levels of *PI3K* were significantly increased in monocrotophos fed flies but fucoidan maintained the PKI3/AKT cell survival pathways in *D*. *melanogaster.* The increased levels of caspase-1 and TNF-α when exposed to dichlorvos lead to neuronal loss in brain tissues (40). The levels of TNF-α and caspase-3 proteins were significantly increased in monocrotophos fed flies but fucoidan maintained the neuroinflammation of brain in flies. A graphical scheme illustrated (Figure 18) some of the proposed mechanisms involved in monocrotophos induced Alzheimer's disease in *D. melanogaster.*

Oxidative stress of the electron transport chain (ETC) to oxygen molecule (O_2) leads to degeneration in AD flies (41). The activities of SOD and CAT were significantly reduced in monocrotophos fed flies but fucoidan maintained normal reactive oxygen species (ROS) production of brain flies. A significant decrease in GSH level was well-known due to the inhibition of GSH synthesis or increased utilization of GSH for detoxification of cypermethrin induced free radicals (42). The levels of GSH and LPO were significantly altered in monocrotophos fed flies but fucoidan maintained normal levels in lipid droplets and protein metabolism in brain. *Drosophila melanogaster* NTs were found to be GABA, glutamate, acetylcholine and monoamines- dopamine, serotonin and histamine related in mammals (43).Glutamate is the excitatory amino acid and the GABA inhibitory amino acid neurotransmitter in central nervous system (44). Upon membrane depolarization induced by action potential, GABA can be released from pre-synaptic vesicles into the synaptic cleft, resulting in the burst increase of GABA concentration in the cleft (45). The level of GABA was significantly reduced and the level of glutamate was significantly increased in

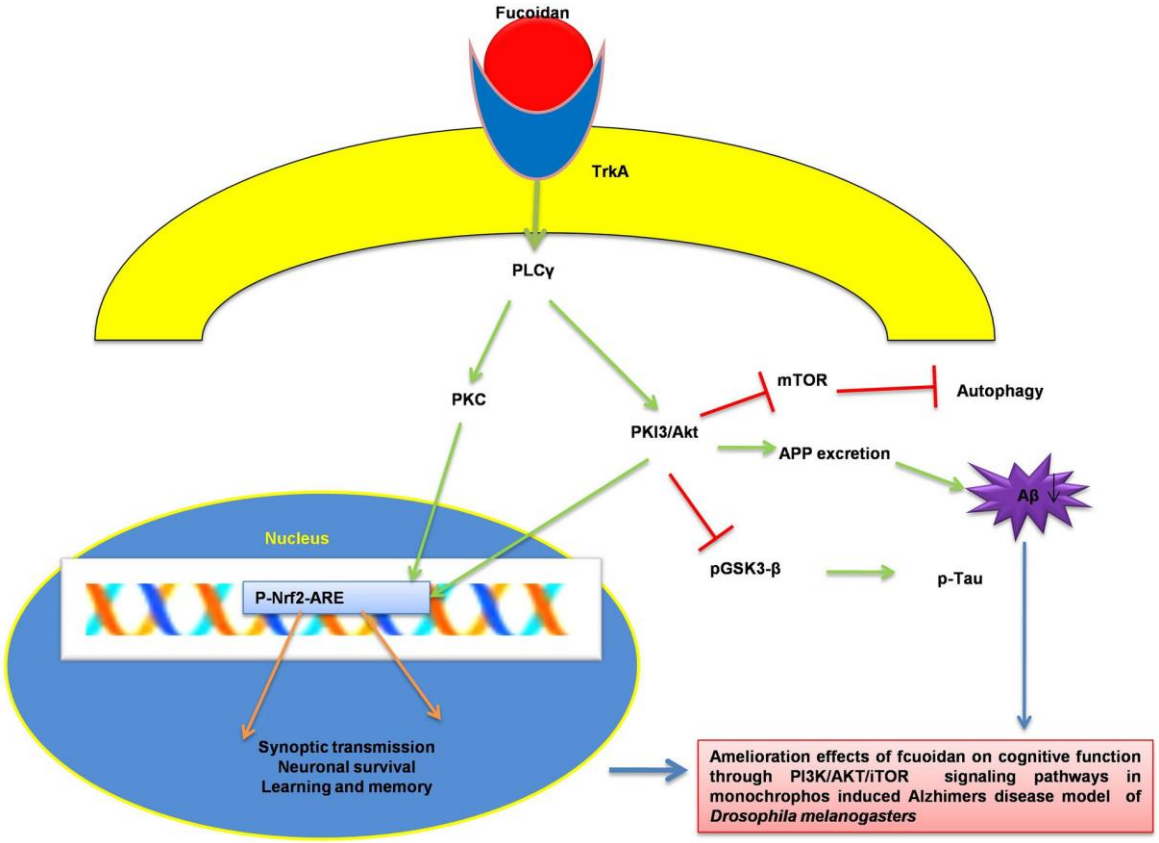

Figure 18. A graphical scheme illustrating some of the proposed mechanisms involved in monocrotophos induced Alzheimer's disease in *D. melanogater* and the probable actions by means of which fucoidan exerts its neuroprotection. A hypothetical scheme of the proposed signaling pathways involved in monocrotophos induced *D. melanogater* and the probable actions by fucoidan and its neuroprotective efficacy. Fucoidan interacts with TrkA signaling pathways that lead to activate the antioxidant (Nrf2-ARE) defense systems in neurons. TrkA activated by fucoidan induces PI3K-Akt signaling pathways that activate Nrf2 and enhances synaptic transmission, neuron survival, and learning and memory. Activated Akt inhibits mTOR to activate autophagy, increases APP excretion and reduces Aβ peptides, and inhibits GSK3β to reduce p-tau. (↑: increases; blue arrows: activate/cause; red lines: inhibit).

monocrotophos fed flies but fucoidan maintained the glutamatergic and GABAergic neuron in brain of *D. melanogater.* The pharmacological therapies are symptomatic that impart clinical benefits on cognitive and functional manifestations of AD. AchE has essential roles in memory and leaning in *D. melanogater (*46, 47). The activity of AchE was significantly increased in monocrotophos fed flies but fucoidan maintained the learning and memory of flies. Octopamine and tyramine regulated the behaviours including courtship, aggressivity and locomotion in flies (48). The levels of OT and TA were significantly reduced on monocrotophos fed flies but fucoidan maintained the locomotoion and aggressive behaviours in flies. The levels of DA and 5-TH were significantly reduced in monocrotophos fed flies but fucoidan maintained the synoptic vesicles trafficking in pathways of brain in *D. melanogater*. In our study *m-RNAs* and protein levels of AchE, β-APP, Tau, PI3K, and BAEC1 were significantly increased (p<0.01) in monocrotophos fed flies but fucoidan maintained neuroprotective action in brain. Fucoidan maintained the mRNA and protein levels in brain as observed in PCR and blotting; it is presumed present that they may regulate Ras/Raf/MAP kinase, PI3K/Akt and PLC-γ signalling pathways in brain. The potential study highlights that fucoidan interacted with Trk-A protein and GSK-3β/Tau, β-APP, Tau, PIK3 and BAEC1 signaling pathways of *D. melagaster..*

6. CONCLUSION AND PERSPECTIVE

Our *In silico* analysis showed that fucoidan targeted pathological proteins by interacting through hydrogen bonding with specific amino acid residue sites and it was found to have good binding energy of -9.3 kcal. In this study, we have demonstrated through *in vitro* experiments that fucoidan had overcome the BBB; it was in nontoxic nature, it acted to treated drug AD when compared to other test compounds. Kinetic studies of the novel selective MAO-A and AchE inhibition activities in PC12 cells showed fucoidan to be a multi-functional anti-AD agent. In addition to *In vivo* studies, the levels *m-RANs* and proteins were significantly altered in monocrotophros fed flies but fucoidan maintained the learning and memory in fed flies. Fucoidan could be considered as a promising candidate to treat AD and it is worthy of further investigation.

7. ACKNOWLEDGMENT

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Abbreviations: AD: Alzheimer disease, NDD: Neurodegenerative diseases, CNS: Central nervous system, BBB: Blood-brain barrier, TNF-α: Tumor necrosis factor alpha, Casp3: caspase 3, Bcl2: B-cell lymphoma 2, PC12: phaeochromocytoma 12, MAO-A: Monoamine oxidase A, MAO-B: Monoamine oxidase B, AchE: Acetylcholinesterase, BuchE: Butyrylcholinesterase, PI3K: Phosphoinositide 3-kinases, TrkA: Tropomyosin receptor kinase A, T Proteins: Tau proteins, BACE-1: Beta-secretase 1, APP: Amyloid precursor protein, LPO: Lipid peroxidation, CAT: Catalase, SOD: Superoxide dismutase, GSH: Glutathione, NTs: Neurotransmitters, GABA: Gamma-aminobutyric acid, Glu: Glutamate, BAs: Biogenic amines, DA: Dopamine, OA: Octopamine, TA: Tryptamine, 5-HT: 5-hydroxytryptamine, Q-RTPCR: Quantitative reverse transcription polymerase chain reaction, Semi-quantitative RTPCR: Semi-quantitative reverse-transcriptase polymerase chain reaction, ELISA: Enzyme-linked immunosorbent assay, MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

Key Words: Molecular docking, fucoidan, monocrotophos, PC12 human neuroblastomal cells, *Drosophila melanogaster* and Alzheimer's disease

Send correspondence to: Savarimuthu Ignacimuthu, Division of vector control, Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, 600034, India, Tel: 044-28178348, Fax: 004-28174644, E-mail: rajivsuba@gmail.com