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RESEARCH ARTICLE

Neuroprotective Effects of Fenugreek Leaf Extract in a *Drosophila* Model of Alzheimer's Disease Expressing Human Aβ-42

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Abstract: ***Introduction*:** Much emphasis has been given to the biological activities of Fenugreek against various diseased conditions. This study investigated the effect of fenugreek leaf extract on behavioural and cognitive function of transgenic *Drosophila* having human Aβ-42 expression in the neurons, herein referred as Alzheimer's disease model flies (AD flies).

***Methods*:** AD flies were exposed to four different doses of fenugreek leaf extract (FE) containing *i.e.,* 0.005, 0.010, 0.015 and 0.02 g/ml for 30 days. Thereafter, behavioural and cognitive assessment was done using climbing ability, activity pattern, aversive phototaxis and odour choice indexes. The life span of different groups of flies was also recorded. The effect of FE on the oxidative stress markers, acetylcholinesterase, monoamine oxidase (MAO) and caspase 3 and 9 activities were determined. The deposition of Aβ-42 aggregates in the brain tissue of the flies was studied by performing immunostaining. Also, the metabolic profile of different groups of flies was studied by performing LC-MS/MS. Compared with control flies, 22 selected metabolites were found to be upregulated and downregulated among transgenic AD flies and FE exposed AD flies compared to control.

***Results*:** The findings of this study showed the neuroprotective role of fenugreek extract, which could be employed for the treatment of Alzheimer's disease. The AD flies exposed to FE showed a dose-dependent postponement in the decline of climbing ability, activity and cognitive impairments. A significant dose dependent increase in the life span was also noticed in the AD flies exposed to FE. A significant reduction in the oxidative stress, acetylcholinesterase, monoamine oxidase, and caspase-3&9 activities was also observed in a dose dependent manner. The results obtained from the immunostaining suggest the reduction in the deposition of Aβ-42 fibril, which was also confirmed by the docking studies showed the energetically favoured interaction useful for inhibiting the acetylcholinesterase and Aβ-42 aggregates.

***Discussion*:** This study demonstrates the neurological potency of fenugreek leaf extract (FE) in a *Drosophila* model of AD due to its antioxidantive, anti-cholinesterase, and neuroprotective properties. Using a combination of behavioral, biochemical, histological, and metabolomic approaches, we evaluated the therapeutic potential of FE in mitigating AD-like symptoms in transgenic flies expressing Aβ-42.

***Conclusion*:** Fenugreek leaf extract may serve as a potential natural remedy for slowing down or alleviating the progression of AD.

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1. INTRODUCTION

Alzheimer's disease (AD) is a type of senile dementia that causes irreversible cognitive impairments through irreversible regression of the brain [1]. The number of people affected by AD could rise to 13.8 million by 2060 if no breakthroughs in medical research are made to cure or slow down the disease development [2]. The progressive neurodegeneration is due to the accumulation of Aβ aggregates, hyperphosphorylated tau protein *i.e.,* neurofibrillary tangles, and loss of cholinergic neurons [2]. The pathology of AD involves a multitude of factors, including cortical atrophy, neuronal loss, neuroinflammation, oxidative stress, and abnormal protein aggregation [3]. Aggregation of amyloid proteins in the tissues of transgenic *Drosophila* fly models, based on the GAL4-UAS system, is capable of inducing the sequential progression of pathological symptoms, similar to that observed in the AD mouse model and AD patients [4]. Patients with severe dementia are incapable to communicate and show walking disability. Similarly, *Drosophila* fly model showing human Aβ-42 in the neurons exhibits loss of climbing ability. The treatment of AD has been limited to a few drugs, as there is no permanent cure. The aggregation of misfolded proteins creates oxidative stress in the brain tissue of AD patients [5]. *Drosophila* serves as an effective model organism for AD research due to its ease of use and its ability to replicate key AD characteristics. These include progressive neuronal degeneration in the eye, vision impairment, rough eye morphology, deficits in learning and behaviour, impaired climbing and flight abilities, reduced mobility, disrupted sleep patterns, and cellular abnormalities, such as protein aggregate formation. Additionally, techniques like chemical mutagenesis and P-element-based screening are instrumental in identifying interacting genes that influence these AD-related traits. With a short life span and easy maintenance, *Drosophila* shares about ~75% conserved homology with human diseases-related genes, which makes it useful for research purposes. In *Drosophila*, there exists an analogous protein to human APP, referred to as APP-like protein (APP-L). Additionally, *Drosophila* possesses orthologs for all components of the γ-secretase complex, albeit with a solitary presenilin and APH gene [4]. A number of studies have been performed to investigate the effect of plant products/extracts using *Drosophila* as a research model [6]. This makes it a valuable model for evaluating potential therapeutic candidates in terms of their predictive efficacy. Molecular and genetic manipulations allow us to study pathophysiological pathways, which helps us to understand complex diseases more comprehensively. Consequently, secondary metabolites or extracts could play a crucial role in reducing brain oxidative stress [6]. A wide range of plant metabolites and phytochemicals showing protective effects against AD has been assessed by Varshney and Siddique [6]. Fenugreek is a widely known herb that has long been used in indigenous medicine to treat various diseases. It is used as an anti-diabetic, hypolipidemic, antimicrobial, and analgesic agent. Additionally, it also contains anti-oxidative, anticancerous, anti-ulcer, anti-fertility and immunomodulatory properties [7]. Further studies have reported its antidepressant, anti-inflammatory effect and support its acetylcholinesterase inhibitory nature. Varshney and Siddique [8] have extensively reviewed the role of bioactive components of fenugreek and their anti-Alzheimer effect on various animal models. The present study was conducted to examine the effect of fenugreek leaf extract on the transgenic *Drosophila melanogaster* expressing Aβ-42 in the neurons. Further, we studied and compared 22 metabolites of the brain of AD flies, AD flies exposed to fenugreek extract and unexposed flies to study the change in the metabolic profiles. Metabolomics quantitatively assesses metabolites that represent changes in genetics, transcription, and protein profiles resulting from disease pathogenesis [8]. Since metabolites are downstream of genomic, epigenomic, and physiological regulation, they serve as a reliable marker of neurodegenerative cellular processes.

2. MATERIALS AND METHODS

2.1. *Drosophila* Stocks

In this study, transgenic fly lines expressing wild-type human Aβ42, *w[1118]; P{w[+mC]=UASAPP.Aβ42.B} m26a*, under UAS control, along with GAL4 drivers (*w[*]; P{w[+mC]=GAL4-elavL}*), sourced from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). For targeted gene expression in *Drosophila*, the GAL4/UAS system has been developed. The bipartite approach involves two components, the responder and the driver (maintained as separate lines). This system controls the expression of the responder gene by binding the GAL4 protein (Yeast transcription factor) to the UAS element [9].

2.2. *Drosophila* Culture and Crosses

Flies were fed on the diet containing corn meal, sugar, agar, and yeast at 25°C (24 ± 1) [10]. Crosses were set accordingly outlined by Ali *et al.* [11]. For 30 days, AD flies were fed different doses of Fenugreek leaf extract mixed into their diet.

2.3. Preparation of Fenugreek Leaf Extract

The leaves of Fenugreek were identified by Prof. M. Badruzzaman Siddiqui of the Department of Botany at Aligarh Muslim University in Aligarh (Specimen Voucher No:31208). A fine powder was prepared by pounding the leaves and allowing them to air dry. Using Soxhlet's apparatus, samples (dry weight 16 grams) were soaked in 300 ml of acetone for 8 hours at 90°C. The extract was analyzed by performing Gas Chromatography Mass Spectrometry (GCMS) at Delhi Test House, New Delhi. The extract concentrations in food were established as 0.005 g/ml, 0.010 g/ml, 0.015 g/ml and 0.02 g/ml.

2.4. Life Span Determination

For the determination of survival of flies, newly eclosed male flies (control and AD flies) were kept in culture vials (10 flies per vial; 3 replicates/group) containing diet. AD and control flies were separately exposed to the diet having varied concentrations, *i.e.*; 0.005 g/ml, 0.010 g/ml, 0.015 g/ml and 0.02 g/ml of fenugreek extract. The AD flies were also exposed separately to 10mM of donepezil hydrochloride in the diet. After every 3rd day, flies were moved to a new diet, and the count of dead flies was taken every three days until all flies had died [12].

2.5. *Drosophila* Climbing Assay

The climbing assay followed the method outlined by Pendleton *et al.* [13]. Flies were kept in an empty glass vial (10.5 cm x 2.5 cm) with a horizontal line marked 8 cm above the bottom (10 flies in a vial). After allowing the flies to acclimate for 10 minutes at room temperature, both control and exposed groups were tested randomly for 10 trials. The data was analysed according to the procedure outlined [11].

2.6. *Drosophila* Activity Pattern

The activity of male flies in all exposed groups was monitored and analyzed by using *Drosophila* Activity Monitor (DAM) (TriTek, USA). The movement of 25 male flies for each group was noted for a period of 36 days.

2.7. Memory and Cognitive Assessment

2.7.1 Aversive Phototaxis Suppression (APS) Assay

For APS assay, the aversive stimuli (Quinine in this experiment) was associated with light using positive phototactic behavior in flies as reported by Ali *et al.* [14]. After 30 days of emergence, male flies (3 replicates/group) were conditioned for the study. In the Y-maze, the flies were first conditioned to acquire short-term memory towards 1 µM quinine. Following the training, the learning behavior of flies was assessed and recorded as PC0 (0h post-conditioning). Following conditioning, the experiment was conducted six hours later, and the results were recorded as PC6 (6 hours after conditioning).

2.7.2 Odour Choice Index

The assay was conducted following the method described by Simonnet *et al.* [15]. The test flies were starved for 16-18 hrs at 25°C prior to the experiment. In one arm of the Y maze, filter papers dipped in propionic acid and in the second arm of the Y maze, filter papers dipped in distilled water were kept. After 30 days of the exposure the experiment was performed for flies of control and treated groups. The number of flies entering each tube of the arm was counted. For each group, five replicates and twenty flies were used. The odour choice index (OCI) was calculated as:

$$OCI = \frac{[\text{Number of flies in tube 1} - \text{Number of flies in tube 2}]}{\text{Total number of flies}}$$

2.8. Preparation of Homogenate for Biochemical Assays

The heads of flies were separated (100 heads/group; five replicates/group), homogenized in Phosphate buffer (0.1M, pH 7.3) and centrifuged at 10,000 g for 10 minutes to obtain a clear supernatant for the biochemical assays.

2.8.1. Estimation of Glutathione (GSH) Content

The colorimetric estimation of glutathione (GSH) was done by using Ellman's reagent (DTNB) according to the method described by Jollow *et al.* [16]. 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant, and 100 µl of DTNB were added for the assay. OD of the mixture was read at 412 nm and expressed as µmoles of GSH/gram tissue.

2.8.2. Estimation of Glutathione-S-transferase (GST) Activity

Glutathione-S-transferase activity was measured by using the method of Habig *et al.* [17]. A mixture containing 500 µl of phosphate buffer, 150 µl of CDNB, 200 µl of reduced glutathione, 50 µl of supernatant, and 10 mM reduced glutathione was added to the reaction. The intensity of the reaction was read at 340 nm, and enzyme activity was reported as µmoles of CDNB conjugates formed per minute per milligram of protein.

2.8.3. Lipid Peroxidation Assay

A method outlined by Ohkawa *et al.* [18] was used to measure lipid peroxidation. 90 µl of sample was heated in a water bath at 90°C for 45 minutes with 5 µl of 10 M butylhydroxy toluene (BHT), 200 µl of 0.67% thiobarbituric acid (TBA), and 600 µl of 1% orthophosphoric acid (OPA) in 105 µl of distilled water. At 535 nm, the intensity of colouration was measured and expressed as µmoles of TBARS formed/h/gram tissue.

2.8.4. Estimation of Protein Carbonyl Content (PCC)

The protein carbonyl content was measured following the method outlined by Hawkins *et al.* [19]. A 250 µl aliquot of the diluted homogenate was combined with an equal volume (250 µl) of 10 mM 2,4-dinitrophenyl hydrazine prepared in 2.5 M HCl. The mixture was vortexed thoroughly and incubated in the dark for 20 minutes. The mixture was thoroughly mixed and incubated at -20°C for 15 minutes with 125 µl of trichloroacetic acid (TCA) at 50% (w/v). The tubes were centrifuged at 8200 g at 4°C for 10 minutes. The pellet obtained was washed twice with ice-cold ethanol:ethyl acetate (1:1). Subsequently, the pellet was dissolved in 6 M guanidine hydrochloride (1 ml), and the intensity of reaction was measured at 370 nm.

2.8.5. Determination of Superoxide Dismutase Activity (SOD) Activity

Superoxide dismutase (SOD) activity was determined using the method described by Marklund [20]. The reaction mixture was prepared by adding 950 µl of 0.1 M phosphate buffer in the 17 µl of the sample, and then pyragallol was added. At 420 nm, OD was read for 3 minutes at 30 seconds interval and the calculated readings were expressed as units/mg protein.

2.8.6. Determination of Catalase (CAT) Activity

Catalase activity was measured as per the procedure of Beers and Sizer's [21] kinetic method by measuring the rate of dismutation of H_2O_2 to water and molecular oxygen in the sample as a function of catalase concentration. The reaction mixture was prepared by adding 650 µl of 0.1 M phosphate buffer, 333 µl of 0.05 M hydrogen peroxide, and 17 µl of the sample. At intervals of 30 seconds, the OD was read at 240 nm for a period of 2 minutes. The catalase activity was expressed as µ moles of H_2O_2 consumed/min/mg protein.

2.8.7. Caspase-3 (Drice) and Caspase-9 (Dronc) Activities

The assay was performed as per the manufacturer's instructions (Bio-Vision, CA, USA). Based on spectrophoto-

metric detection of chromophore p-nitroanilide (pNA), the assay was conducted using DEVD-pNA and LEHD-pNA, tetrapeptide substrates targeted by caspase-3 and caspase-9. After mixing 50 µl of homogenate with 50 µl of chilled cell lysis buffer, the assay mixture was incubated on ice for 10 minutes. After 10 min, 50 µl of 2X reaction buffer, which included 10 mM DTT, was mixed with 200 µl of the substrate (DEVD-pNA for Drice or IETD-pNA for Dronc) and allow it to sit for 1.5 hours at 37°C. The optical density (OD) was then measured at 405 nm.

2.8.8. Acetylcholinesterase (AChE) Activity

A method described by Ellman *et al.* [22] was used to determine acetylcholinesterase activity. In this reaction, acetylthiocholine is hydrolyzed by AChE, resulting in thiocholine and acetate. As a result, thiocholine reduces Dithiobis-nitrobenzoate, releasing nitrobenzoate. The reaction mixture for the experiment was prepared by combining 100 µl of the sample, 650 µl of 0.1 M phosphate buffer, and 100 µl of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). 10 µl of acetylthiocholine was added to the mixture, and the reaction was monitored for 3 minutes at 412 nm.

2.8.9. Estimation of Monoamine Oxidase (MAO)

Monoamine oxidase activity was measured following the method outlined by McEwen [23]. A mixture of 400 µl 0.1M phosphate buffer (pH 7.4), 1300 µl distilled water, 100 µl benzylamine hydrochloride, and 200 µl brain homogenate was used in the assay. The mixture was incubated at room temperature for 30 minutes. Subsequently, 1 ml of 10% perchloric acid was added and centrifuged at 1500g for 10 minutes. The absorbance was measured at 280 nm.

2.9. Immunohistochemistry

A method described by Palladino *et al.* [24] was used to isolate the fly heads and prepare paraffin sections. For immunohistochemical detection of amyloid aggregates, paraffin-embedded head sections were incubated with primary antibody (1:200 dilutions of Rabbit monoclonal Aβ42 antibody from Merck) in a humidified chamber for 12 hours at 4ºC. At room temperature for two hours, the slides were washed with PBS containing 2% BSA for 5 minutes, and incubated with secondary antibody (1:1000 dilutions of Goat anti-Rabbit IgG, alkaline phosphatase conjugate from Merck, USA). For the final wash, PBS was used containing 2% BSA for 5 minutes. To visualize the enzyme-labelled Goat anti-Rabbit IgG 5-Bromo-4-chloro-3-indolyl phosphate-Nitro blue tetrazolium chloride (BCIP-NBT) was used. After mounting in DPX, the slides were examined under a microscope. Aβ42 aggregate were quantified by outlining each plaque area using Image-J software.

2.10. Molecular Docking Study

The molecular docking studies were performed using an interactive protein docking and molecular superposition program called HEX 8.0.0 that calculated and displayed various docking modes [25]. CHEMSKETCH (http://www.acdlabs.com) was used to draw the molecular structure of 2-Myristynoyl pantetheine, and OPENBABEL (http://www.vcclab.org/lab/babel/) was used to convert the molecular structure from Mol to PDB format. From the protein data bank (http://www.rcsb.org./pdb) it was possible to determine the structure of the acetylcholinesterase and Aβ-42 with the PDB ID: 2ONV. The docked pose as well as the interactions between the 2-Myristynoyl pantetheine and Aβ-42 were visualized using CHIMERA (www.cgl.ucsf.edu/ chimera) and PyMol (http://pymol.sourceforget.net/) molecular graphics programs.

2.11. LC-MS/MS

The heads of 250 adult *Drosophila* flies were homogenized in 300 µl of homogenizing solution consisting of 150 µl of methanol and 150 µl of autoclaved Mili-Q water. Centrifuged the mixer at 13,000 rpm for 10 minutes at 4^{o}C. The collected supernatant was passed through 2-µm filter before being analyzed by LC-MS/MS. Instrumentation consisted of a Qexactive (thermoscientific) was used for MS which had a heated electrospray ionization (HESI). At 3200 Voltage source, the sheath gas 30 L/min, AUX gas 10 L/min, swipe gas 0, the ion transfer tube gas was 325^{o}C. The vapourizer temperature was 350^{o}C, S-lens RF level was 50. AGC target-1e6 resolution was 70,000 and the scan range was kept 100-1500 M/Z. The analysis was done was by using a software Thermofisher Scientific Compound Discoverer 3.3. Chromatography was performed on dionex ultimate 3000, thermoscientific on Hypersil C-18 column (2.1 mm X 100 mm, 1.9 µM) using Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile) [26]. The injectable volume was about 15 µl with a flow rate of 200 µl/min. The column temperature was 25^{o}C and the total running time was 12.2 minutes.

2.12. Statistical Analysis

GraphPad Prism software (version 5.0) was used for analysis of the data using one way analysis of variance (ANOVA) and post hoc Tukey test. Statistical significance was set at $p<0.05$. The results were expressed as mean ± SEM. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were done by MetaboAnalystR 6.0 (http://www.metaboanal yst.ca/). Also, volcano plot analysis was done by MetaboAnalystR 6.0 (http://www.metaboanal yst.ca/). Pathway analysis, metabolite-metabolite interaction network, was drawn by MetaboAnalystR 6.0 software and the data were integrated and visualized using Cytoscape 3.6.0 software.

3. RESULTS

The GC-MS analysis of the leaf extract of *T. foenum* revealed the presence of 2-Myristynoyl pantetheine, Pyrano[4,3-b]benzopyran-1,9-dione, Tridecanoic acid, 3-Hexen-2-one, 2-Pentanone, 4-Hydroxy-4-methyl, 2-Myristynoyl pantetheine, 2-Hexadecanol, N,N-Bis(Carbobenzyloxy)-lysine methyl(ester), Retinol and Cyclobarbital. 2-Myristynoyl pantetheine was found to be the most abundant compound in the extract (Table **S1**). The lifespan results are presented in (Fig. **S1**), revealing that on day 9, AD flies showed a 1.16 fold reduction in survival compared to control flies (Fig. **S1**; $p < 0.05$). Exposure to 0.005 g/ml of FE did not significantly improve the survival of AD flies (Fig. **S1**; $p < 0.05$). Similarly, no significant changes were observed in

AD flies treated with 0.010, 0.015, or 0.02 g/ml of FE (Fig. **S1**; $p < 0.05$). By day 15, AD flies exhibited a significant 1.20 fold reduction in survival relative to control flies (Fig. **S1**; $p < 0.05$). At this time point, treatment with 0.005 g/ml of FE did not significantly extend lifespan (Fig. **S1**; $p < 0.05$), whereas exposure to 0.010, 0.015, and 0.02 g/ml of FE resulted in dose-dependent increases in survival by 1.03, 1.08, and 1.08 folds, respectively (Fig. **S1**; $p < 0.05$). However, no significant differences were observed between untreated AD flies and those exposed to 0.1 mM DH (Fig. **S1**; $p < 0.05$). On day 21, survival of AD flies decreased by 1.42 fold compared to control flies (Fig. **S1**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly increased survival by 1.04, 1.20, 1.26, and 1.31 fold, respectively, compared to untreated AD flies (Fig. **S1**; $p < 0.05$). Additionally, exposure to 0.1 mM DH resulted in a 1.11 fold increase in survival relative to untreated AD flies (Fig. **S1**; $p < 0.05$). By day 27, AD flies showed a 2.60 fold decrease in survival compared to control flies (Fig. **S1**; $p < 0.05$). Treatments with 0.005, 0.010, 0.015, and 0.02 g/ml of FE led to significant survival improvements of 1.39, 1.90, 2.00, and 2.30 fold, respectively, compared to untreated AD flies (Fig. **S1**; $p < 0.05$). Exposure to 0.1 mM DH also significantly increased survival by 1.69 fold compared to untreated AD flies (Fig. **S1**; $p < 0.05$). On day 33, survival of AD flies was reduced by 12.6 fold compared to control flies (Fig. **S1**; $p < 0.05$). However, treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly improved survival by 3.33, 3.83, 8.83, and 11 fold, respectively, compared to untreated AD flies (Fig. **S1**; $p < 0.05$). Flies exposed to 0.1 mM DH showed a 7.16 fold increase in survival compared to untreated AD flies (Fig. **S1**; $p < 0.05$). By day 39, all AD flies, as well as those treated with 0.005 and 0.010 g/ml of FE, had died (Fig. **S1**). AD flies treated with 0.015 and 0.02 g/ml of FE survived up to 45 and 51 days, respectively (Fig. **S1**; $p < 0.05$), whereas control flies survived for 63 days (Fig. **S1**; $p < 0.05$).

No notable differences in the activity levels of the flies were observed up to the 12th day. From days 13 to 18, the activity of AD flies significantly declined by 1.37 fold compared to control flies (Fig. **S2**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE resulted in a dose-dependent improvement in activity by 1.07, 1.06, 1.00, and 1.01 fold, respectively, compared to untreated AD flies (Fig. **S2**; $p < 0.05$). Between days 19 and 24, AD flies exhibited a 2 fold reduction in activity compared to controls (Fig. **S2**; $p < 0.05$). Exposing AD flies to 0.005, 0.010, 0.015, and 0.02 g/ml of FE led to significant dose-dependent improvements in activity by 1.13, 1.12, 1.25, and 1.58 fold, respectively, compared to untreated AD flies (Fig. **S2**; $p < 0.05$). From days 25 to 30, the activity of AD flies decreased by 4 fold relative to control flies (Fig. **S2**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly enhanced activity by 1.19, 1.38, 1.55, and 2.02 fold, respectively, in a dose-dependent manner compared to untreated AD flies (Fig. **S2**; $p < 0.05$). Between days 31 and 36, AD flies showed a 6.11 fold reduction in activity compared to controls (Fig. **S2**; $p < 0.05$). FE treatment at 0.005, 0.010, 0.015, and 0.02 g/ml resulted in dose-dependent improvements of 1.43, 2.02, 1.96, and 2.03 fold, respectively, compared to untreated AD flies (Fig. **S2**; $p < 0.05$).

No significant improvement in memory loss was observed in AD flies at 6 hours post-treatment (Fig. **S3**; $p < 0.05$). However, AD flies exposed to 0.005, 0.010, 0.015, and 0.02 g/ml of FE showed significant memory improvement by 1.59, 1.58, 1.97, and 2.00 folds, respectively, compared to untreated AD flies (Fig. **S3**; $p < 0.05$). Additionally, AD flies treated with 0.1 mM donepezil hydrochloride displayed a 3.12 fold improvement in memory at 6 hours (Fig. **S3**; $p < 0.05$). The OCI in AD flies decreased significantly by 12.6 fold compared to controls (Fig. **S4**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly increased OCI by 1.6, 4.6, 6.2, and 8.0 folds, respectively, compared to untreated AD flies (Fig. **S4**; $p < 0.05$). AD flies exposed to 0.1 mM donepezil hydrochloride showed a 7.2 fold improvement in OCI relative to untreated AD flies (Fig. **S4**; $p < 0.05$).

Caspase-3 activity in AD flies was significantly elevated by 4.22 fold compared to control flies (Fig. **S5**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE resulted in a significant reduction in caspase-3 activity by 1.11, 1.20, 1.41, and 1.75 folds, respectively, compared to untreated AD flies (Fig. **S5**; $p < 0.05$). Additionally, exposure to 0.1 mM DH significantly reduced caspase-3 activity by 2.49 fold compared to untreated AD flies (Fig. **S5**; $p < 0.05$). Similarly, caspase-9 activity in AD flies showed a significant increase of 4.07 fold compared to control flies (Fig. **S6**; $p < 0.05$). Treatment of AD flies with 0.005, 0.010, 0.015, and 0.02 g/ml of FE led to a dose-dependent significant reduction in caspase-9 activity by 1.15, 1.30, 1.50, and 1.77 fold, respectively, compared to untreated AD flies (Fig. **S6**; $p < 0.05$). Exposure to 0.1 mM DH resulted in a significant 2.28 fold decrease in caspase-9 activity compared to untreated AD flies (Fig. **S6**; $p < 0.05$).

The climbing ability of AD flies was significantly reduced by 4.33 fold compared to control flies (Fig. **1**; $p < 0.05$). When exposed to 0.005, 0.010, 0.015, and 0.02 g/ml of FE, AD flies showed significant improvements in climbing ability by 1.04, 1.37, 1.97, and 2.54 fold, respectively, compared to untreated AD flies (Fig. **1**; $p < 0.05$). Additionally, AD flies treated with 0.1 mM DH exhibited a 3.08 fold improvement in climbing ability compared to untreated AD flies (Fig. **1**; $p < 0.05$).

The levels of TBARS in AD flies were significantly elevated by 3.51 fold compared to control flies (Fig. **2a**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE resulted in significant reductions in TBARS levels by 1.15, 1.34, 1.52, and 1.67 fold, respectively, compared to untreated AD flies (Fig. **2a**; $p < 0.05$). Similarly, exposure to 0.1 mM DH significantly decreased TBARS levels by 1.38 fold compared to untreated AD flies (Fig. **2a**; $p < 0.05$). The GSH content in AD flies was significantly reduced by 4.37 fold relative to control flies (Fig. **2b**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE led to a dose-dependent increase in GSH levels by 1.24, 1.56, 1.97, and 2.60 fold, respectively, compared to untreated AD flies (Fig. **2b**; $p < 0.05$). Additionally, exposure to 0.1 mM DH significantly elevated GSH content by 3.30 fold compared to untreated AD flies (Fig. **2b**; $p < 0.05$). The activity of GST in AD flies was significantly elevated by 2.80 fold compared to control flies (Fig. **2c**; $p < 0.05$). However, treatment with

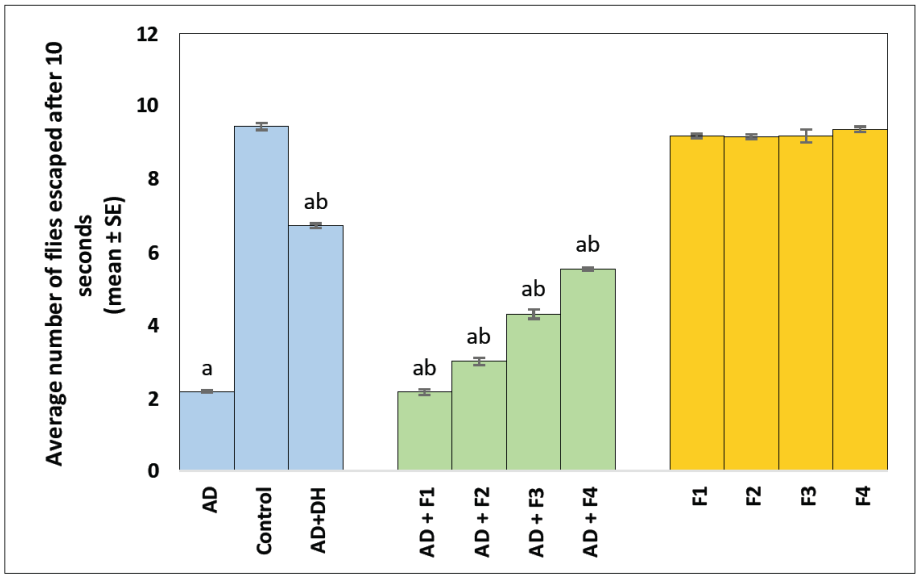


Fig. (1). Effect of Fenugreek extract on the climbing ability of AD and control flies. (F1 = 0.005 g/ml, F2 = 0.010 g/ml, F3 = 0.015 g/ml, F4 = 0.02 g/ml; AD = AD flies; DH = 0.1 mM Donepezil hydrochloride). The flies were allowed to feed on the diet supplemented with FE for 30 days and then assayed for climbing ability. (a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to AD). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly decreased GST activity by 1.09, 1.17, 1.29, and 1.47 folds, respectively, compared to untreated AD flies (Fig. **2c**; $p < 0.05$). Similarly, exposure to 0.1 mM DH reduced GST activity by 1.86 fold compared to untreated AD flies (Fig. **2c**; $p < 0.05$). The SOD activity in AD flies was significantly higher, showing a 3.15 fold increase compared to control flies (Fig. **2d**; $p < 0.05$). Upon exposure to 0.005, 0.010, 0.015, and 0.02 g/ml of FE, SOD activity significantly decreased by 1.13, 1.22, 1.47, and 1.80 folds, respectively, compared to untreated AD flies (Fig. **2d**; $p < 0.05$). Treatment with 0.1 mM DH resulted in a significant 1.42 fold reduction in SOD activity compared to untreated AD flies (Fig. **2d**; $p < 0.05$). Catalase activity in AD flies was significantly elevated by 2.53 fold compared to control flies (Fig. **2e**; $p < 0.05$). Treatments with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly reduced catalase activity in a dose-dependent manner by 1.12, 1.27, 1.45, and 1.67 folds, respectively, compared to untreated AD flies (Fig. **2e**; $p < 0.05$). Exposure to 0.1 mM DH significantly decreased catalase activity by 1.46 fold relative to untreated AD flies (Fig. **2e**; $p < 0.05$). The protein carbonyl (PC) content in AD flies showed a 3.98 fold increase compared to control flies (Fig. **2f**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly decreased PC levels by 1.08, 1.18, 1.27, and 1.44 fold, respectively, compared to untreated AD flies (Fig. **2f**; $p < 0.05$). Exposure to 0.1 mM DH resulted in a significant 2.20 fold reduction in PC content compared to untreated AD flies (Fig. **2f**; $p < 0.05$).

The activity of Acetylcholinesterase (AChE) in AD flies was reduced by 1.13 fold compared to control flies (Fig. **3a**; $p < 0.05$). When AD flies were treated with 0.005, 0.010, 0.015, and 0.02 g/ml of FE, a dose-dependent and significant reduction in AChE activity was observed by 1.17, 1.29, 1.51, and 1.75 folds, respectively, compared to untreated AD flies (Fig. **3a**; $p < 0.05$). In control flies, exposure to the same concentrations of FE resulted in significant, dose-dependent decreases in AChE activity by 1.07, 1.12, 1.27, and 1.45 folds compared to unexposed control flies (Fig. **3a**; $p < 0.05$). Additionally, AD flies treated with 0.1 mM DH exhibited a significant reduction in AChE activity by 2.29 fold compared to untreated AD flies (Fig. **3a**; $p < 0.05$). The activity of monoamine oxidase (MAO) was significantly elevated in AD flies by 7.29-fold compared to control flies (Fig. **3b**; $p < 0.05$). Treatment of AD flies with 0.005, 0.010, 0.015, and 0.02 g/ml of FE significantly reduced MAO activity in a dose-dependent manner by 1.35, 1.92, 1.98, and 2.08 folds, respectively, compared to untreated AD flies (Fig. **3b**; $p < 0.05$). Furthermore, exposure to 0.1 mM DH led to a significant 3.83 fold decrease in MAO activity compared to untreated AD flies (Fig. **3b**; $p < 0.05$).

The wire form model of acetylcholinesterase is shown in Fig. (**4a, b** and **c**). The docked model of 2-Myristynoyl pantetheine with acetylcholinesterase is shown in (Fig. **4d**), and various docked pose are depicted in Fig. (**4e**). The molecular docking results indicate that 2-Myristynoyl pantetheine adopts its most energetically favorable configuration, allowing it to effectively fit into the twisted structure of acetylcholinesterase (Fig. **4f**). 2-Myristynoyl pantetheine stays stabilized in the docked conformation through hydrophobic interactions, hydrogen bonding, and van der Waals forces with surrounding amino acids. Arginine interacts with oxygen (ARG 467.A 2HH2···het O2.12Å), hydrogen (ARG 467.A 1HH2···het H2.24Å) and nitrogen (ARG 467.A 1HH1··· het N 3.39Å). Sulphur atom interacts with lysine (LYS 478.A 1HZ···het S3.34Å) and histidine (Fig. **4f**; Table **1**). The capped stick and surface covered representation of Aβ-42 is shown in Fig. (**4g**) (i & ii). Aβ (or Abeta) peptides, which are 40 or 42 amino acids in length, serve as the primary component of amyloid plaques in the brains of individuals with AD. These peptides adopt a highly organized structure referred to as the cross-β spine or amyloid. The

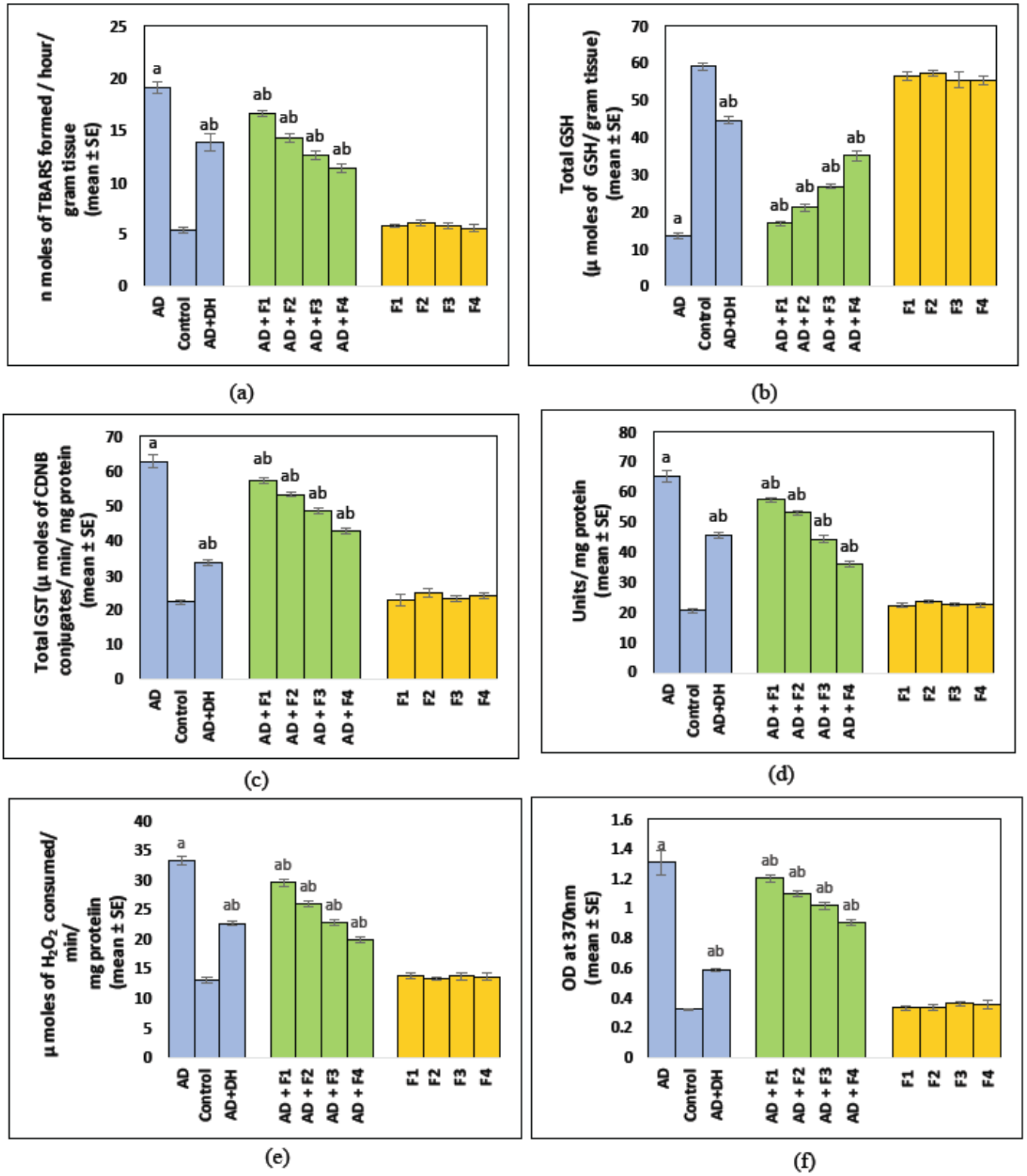
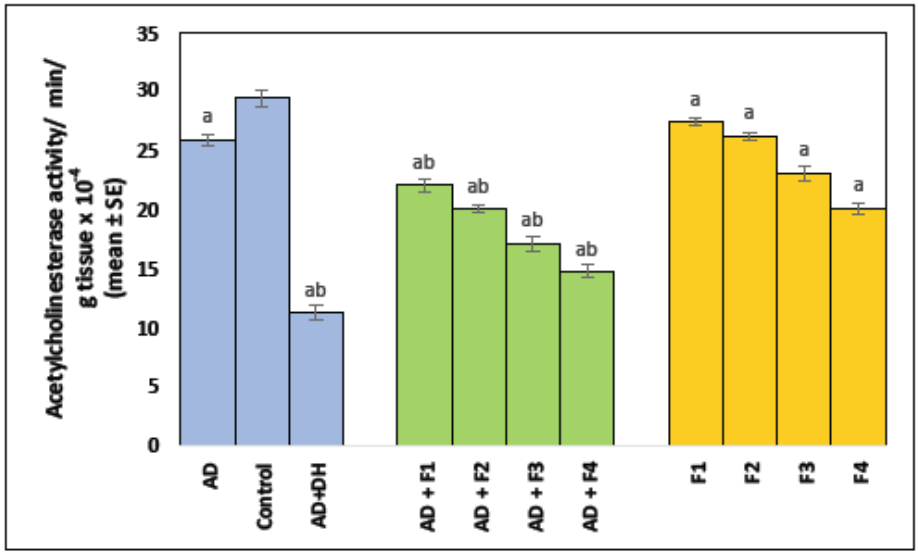
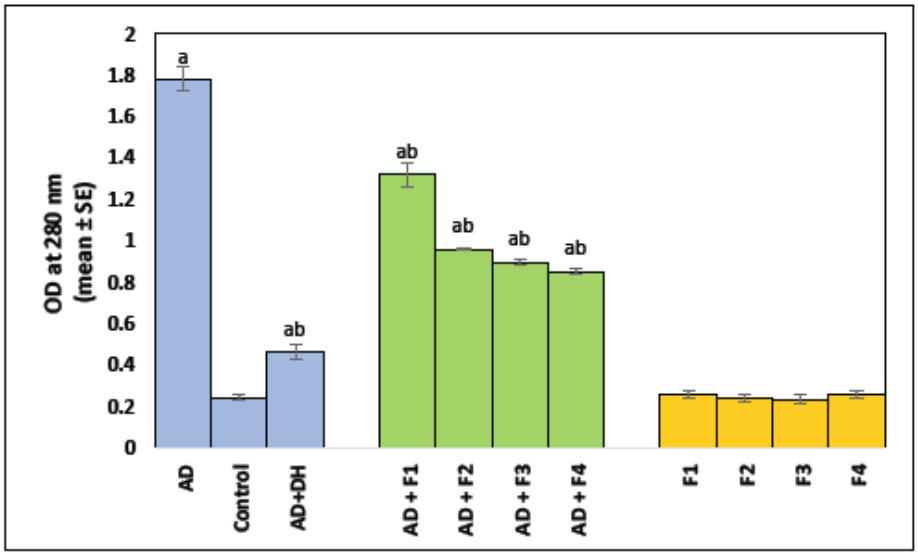


Fig. (2). Effect of Fenugreek extract on lipid peroxidation (LPO) (**a**), reduced glutathione (GSH) content (**b**), glutathione-S-transferase (GST) activity (**c**), Superoxide dismutase activity (SOD) (**d**), Catalase activity (**e**), and protein carbonyl content (PCC) (**f**), in the brains of AD and control flies. (F1 = 0.005 g/ml, F2 = 0.010 g/ml, F3 = 0.015 g/ml, F4 = 0.02 g/ml; AD = AD flies; DH = 0.1 mM Donepezil hydrochloride). The flies were allowed to feed on the diet supplemented with FE for 30 days and then assayed for the given parameters (a-significant at $p<0.05$ compared to control; b-significant at $p<0.05$ compared to AD). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*



(a)



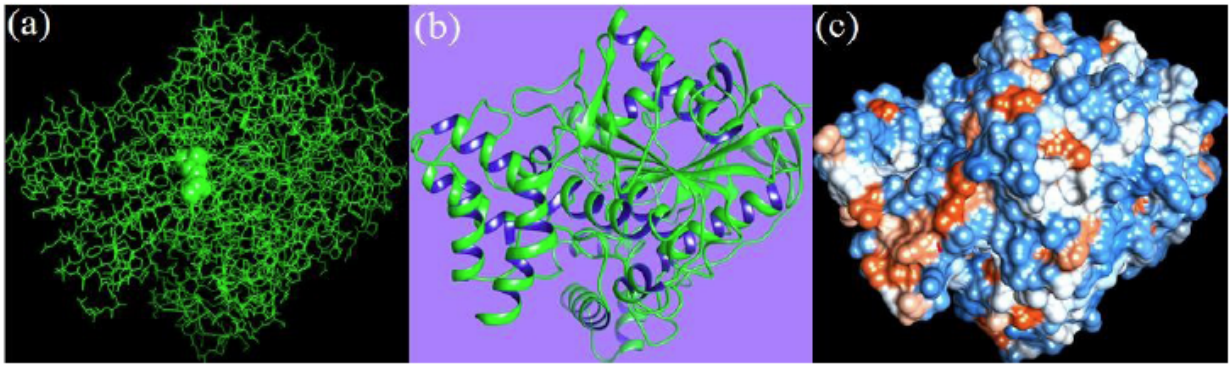
(b)

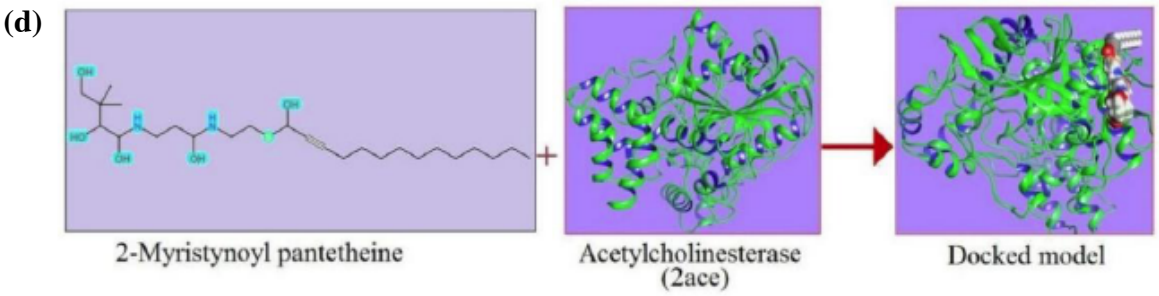
Fig. (3). Effect of FE on the activity of acetylcholinesterase activity (**a**) and monoamine oxidase activity (**b**) in the brains of flies. (F1 = 0.005 g/ml, F2 = 0.010 g/ml, F3 = 0.015 g/ml, F4 = 0.02 g/ml; AD = AD flies; DH = 0.1 mM Donepezil hydrochloride). The flies were allowed to feed on the diet supplemented with FE for 30 days and then assayed for the acetylcholinesterase activity and monoamine oxidase activity (a- significant at $p < 0.05$ compared to control; b- significant at $p < 0.05$ compared to AD). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

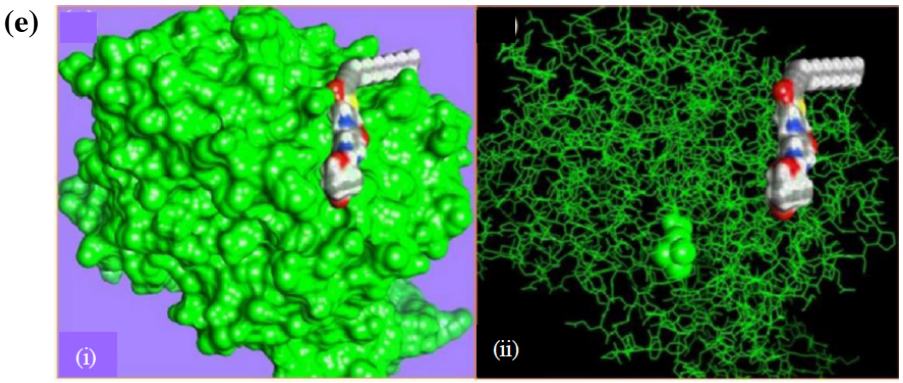
docked model of configuration of 2-Myristynoyl pantetheine with Aβ-42 is shown in Fig. (**4h**) (Fig. **4i** & Table **1**). Fig. (**4g**) (i) shows the capped stick model of Aβ42, while Fig. (**4g**) (ii) represents the surface covered model of Aβ42, respectively. Similarly, the structure of 2-Myristynoyl pantetheine and its interaction with the protein, leading to the formation of the docked complex model, is shown in Fig. (**4h**). While, (Fig. **4i**), represents the various non-covalent interactions and H-bonds established in the docked model. Table **1**, demonstrates the interactions of the different amino acid residue of Aβ42 with the 2-Myristynoyl pantetheine. From the given table, it is clear that valine, an amino acid of Aβ42 binds with the carbon of the (VAL 3.A H ··· het C 2.57 Å). Glycine and isoleucine also make covalent bonds with carbon. Isoleucine and alanine makes non-covalent interactions with the oxygen atom of the 2-Myristynoyl pantetheine. Similarly, sulphur interacts with Isoleucine (ILE 5.A O··· het S 2.92 Å) (Table **1**).

Table 1. H-bondings and non-covalent interactions between amino acid residue of acetylcholinesterase and 2-Myristynoyl pantetheine and Aβ-42.

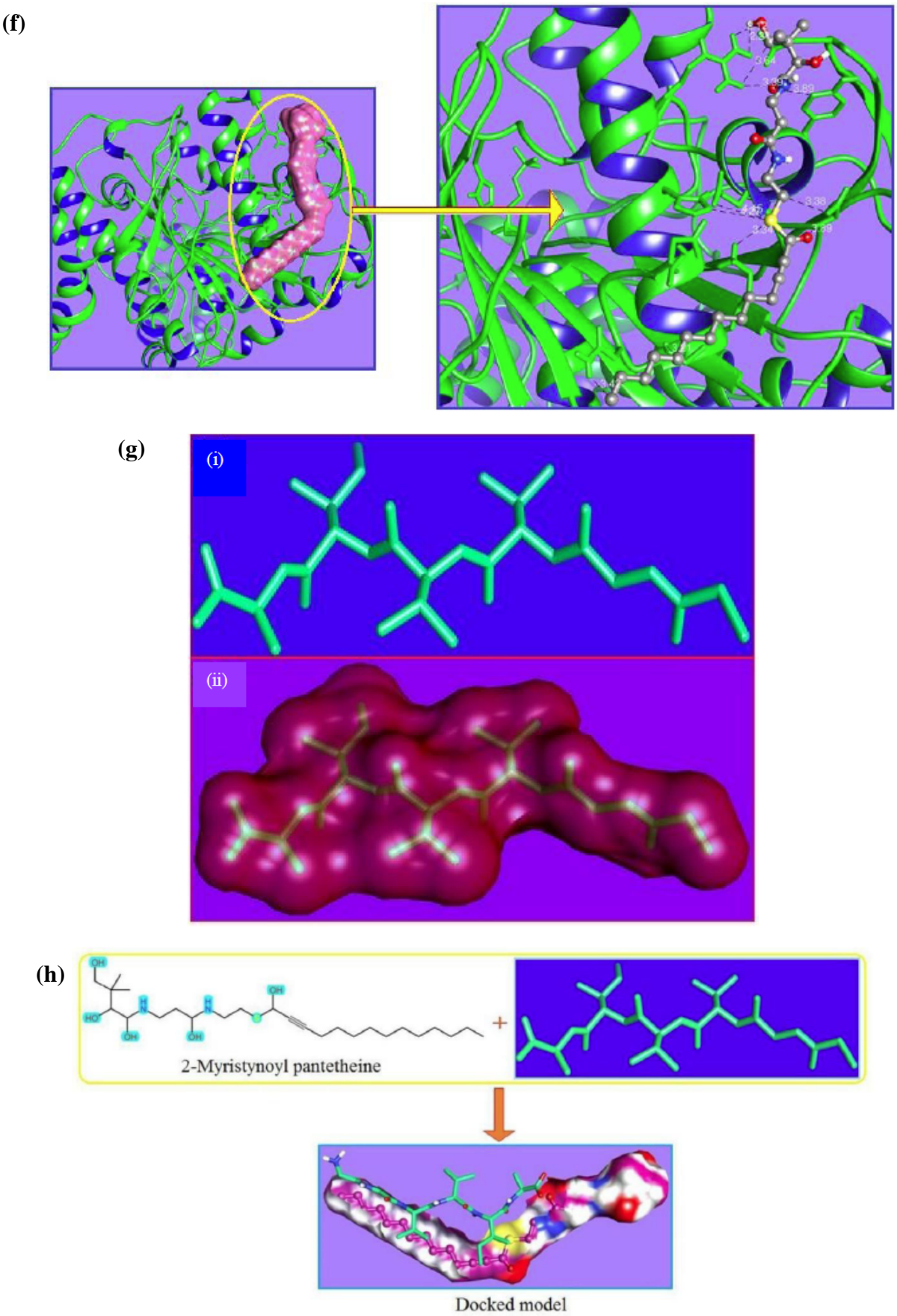
2-Myristynoyl Pantetheine+Acetylcholinesterase (2ACE)				2-Myristynoyl Pantetheine+Aβ42 (2ONV)		
S. No.	**ATOM 1**	**ATOM 2**	**Distance (Å)**	**ATOM 1**	**ATOM 2**	**Distance (Å)**
1.	ARG 467.A 2HH2	1.het O	2.12	VAL 3.A H	1.het C	2.57
2.	ARG 467.A 1HH2	1.het H	2.24	GLY 1.A O	1.het C	2.63
3.	ARG 467.A 1HH1	1.het N	3.39	ILE 5.A O	1.het S	2.92
4.	ARG 467.A 1HH1	1.het C	3.64	ILE 5.A CD1	1.het C	2.95
5.	TYR 137.A CE1	1.het O	3.89	ALA 6.A OXT	1.het O	3.51
6.	LYS 478.A 1HZ	1.het S	3.34	ILE 5.A CD1	1.het O	3.78
7.	GLU 140.A CB	1.het O	3.89	ILE 5.A CB	1.het S	3.25
8.	THR 479.A HG1	1.het C	3.21	VAL 3.A CB	1.het C	3.22
9.	ASN 481.A 1HD2	1.het C	3.42	GLY 1.A 3H	1.het C	3.79
10.	HIS 471.A CE1	1.het S	4.27	ALA 6.A CA	1.het N	4.29







(Fig. 4) Contd….



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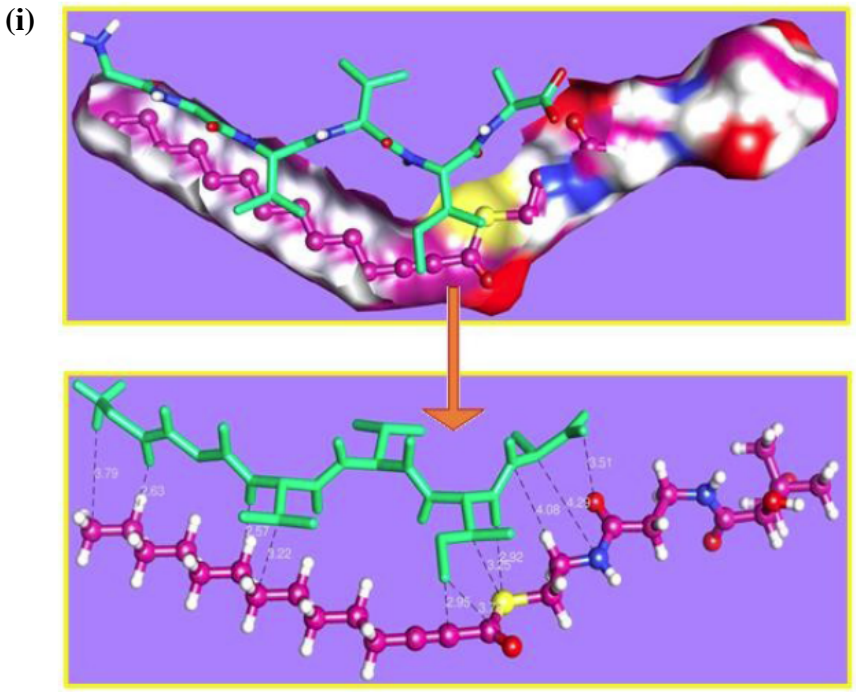


Fig. (4). (**a**) Wire form model of Acetylcholinesterase (**b**) Curled and coiled structure of acetylcholinesterase (**c**) Surface covered representation of the acetylcholinesterase. (**d**) Docked model of 2-Myristynoyl pantetheine with acetylcholinesterase. (**e**) Various docked pose of the 2-Myristynoyl pantetheine - acetylcholinesterase interaction. (**f**) Several non-covalent interactions and hydrophobic interactions exhibited by docked model via bonding between 2-Myristynoyl pantetheine and acetylcholinesterase. (**g**) (i) Capped stick representation of the protein Aβ42 (ii) Surface covered representation of the protein Aβ42. (**h**) Docked model of 2-Myristynoyl pantetheine with Aβ42. (**i**) Molecular docked model exhibiting H-bonding, hydrophobic and non-covalent interactions between 2-Myristynoyl pantetheine and Aβ42 fibril. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

The immunohistochemistry results are presented in Fig. (**5a-f**). Prominent Aβ-42 aggregates were observed in the brains of AD flies (Fig. **5a**,**b**) compared to control flies. A dose-dependent reduction in Aβ-42 aggregate formation was noted in the brains of flies treated with FE (Fig. **5c-f**). Quantitative analysis of Aβ-42 aggregates in AD fly brains is shown in Fig. (**5g**). AD flies exhibited a 310 fold increase in Aβ-42 aggregates compared to controls (Fig. **5g**; $p < 0.05$). Treatment with 0.005, 0.010, 0.015, and 0.02 g/ml of FE resulted in a dose-dependent reduction in Aβ-42 aggregates by 2.80, 4.14, 11.11, and 50 fold, respectively, compared to untreated AD flies (Fig. **5g**; $p < 0.05$).

The results of the metabolic study are presented in Fig. (**6a-v**). A total of 22 metabolites were analyzed from the brain homogenate (Table **S2**). Acetylcholine levels in AD flies were significantly reduced by 3.6 fold compared to control flies (Fig. **6a**; $p < 0.05$). Treatment with FE led to a 2.96 fold increase in acetylcholine levels in AD flies compared to untreated AD flies (Fig. **6a**; $p < 0.05$). Glutathione levels were significantly decreased by 2.32 fold in the brain homogenate of AD flies compared to controls (Fig. **6b**; $p < 0.05$), while FE treatment resulted in a 2.56 fold increase in glutathione levels (Fig. **6b**; $p < 0.05$). Adenosine 5-monophosphate levels were reduced by 1.97 fold in AD flies compared to controls (Fig. **6c**; $p < 0.05$), but exposure to FE caused a 2.63 fold increase (Fig. **6c**; $p < 0.05$). Similarly, N-acetyl-L-aspartic acid levels were decreased by 1.33 fold in AD flies compared to controls (Fig. **6d**; $p < 0.05$), and FE exposure increased these levels by 1.15 fold (Fig. **6d**; $p < 0.05$). Cytidine levels showed a 1.43 fold reduction in AD flies compared to controls (Fig. **6e**; $p < 0.05$), with a 2.54 fold increase following FE treatment compared to AD flies (Fig. **6e**; $p < 0.05$). Carnitine levels were reduced by 1.71 fold in AD flies compared to controls (Fig. **6f**; $p < 0.05$), but treatment with FE elevated these levels by 1.46 fold (Fig. **6f**; $p < 0.05$). Gamma-aminobutyric acid (GABA) levels were significantly decreased by 2.09 fold in AD flies compared to controls (Fig. **6g**; $p < 0.05$), while FE exposure led to a 3.10 fold increase in GABA levels (Fig. **6g**; $p < 0.05$). Uric acid levels in AD flies were reduced by 1.97 fold compared to controls (Fig. **6h**; $p < 0.05$), but treatment with FE resulted in a 1.52 fold increase (Fig. **6h**; $p < 0.05$). Hypoxanthine levels were diminished by 2.31 fold in AD flies compared to controls (Fig. **6i**; $p < 0.05$), while FE treatment elevated these levels by 3.24 fold (Fig. **6i**; $p < 0.05$). Xanthurenic acid levels showed a 1.25 fold decrease in AD flies compared to controls (Fig. **6j**; $p < 0.05$), with FE exposure leading to a 1.52 fold increase (Fig. **6j**; $p < 0.05$). Levels of xanthine, taurine, inosine, allopurinol, and pantothenic acid were significantly reduced by 2.76, 1.68, 2.00, 1.92, and 1.35 folds, respectively, in AD flies compared to control (Fig. **6k-p**; $p < 0.05$). FE treatment significantly increased the levels of these metabolites by 7.93, 2.11, 1.66, 2.45, 2.37, and 2.50 folds, respectively, in AD flies (Fig. **6k-p**; $p < 0.05$). Phosphocholine levels were elevated by 1.56 fold in AD flies compared to controls (Fig. **6q**; $p < 0.05$), but FE treatment reduced these levels by 1.19 folds compared to untreated AD flies

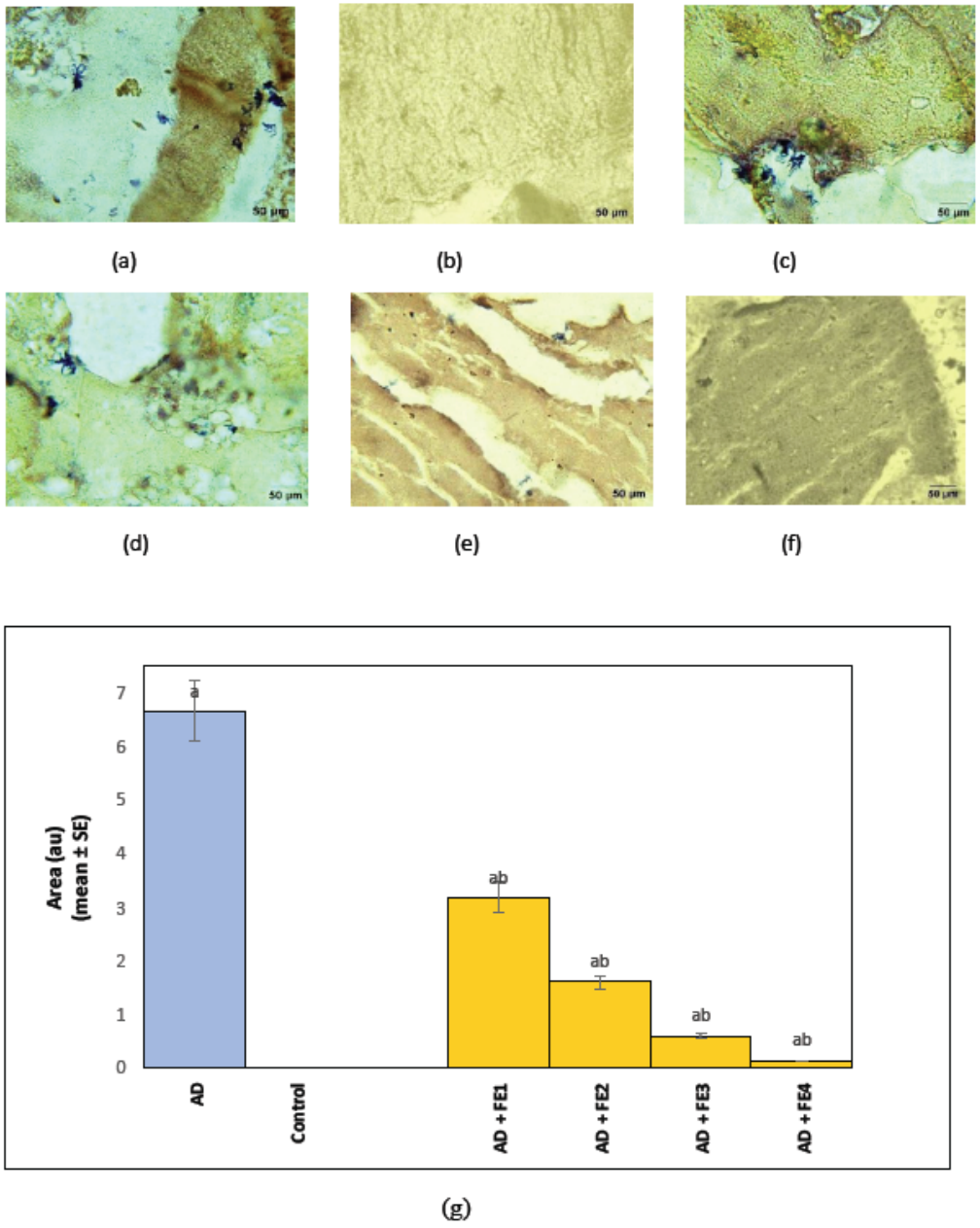
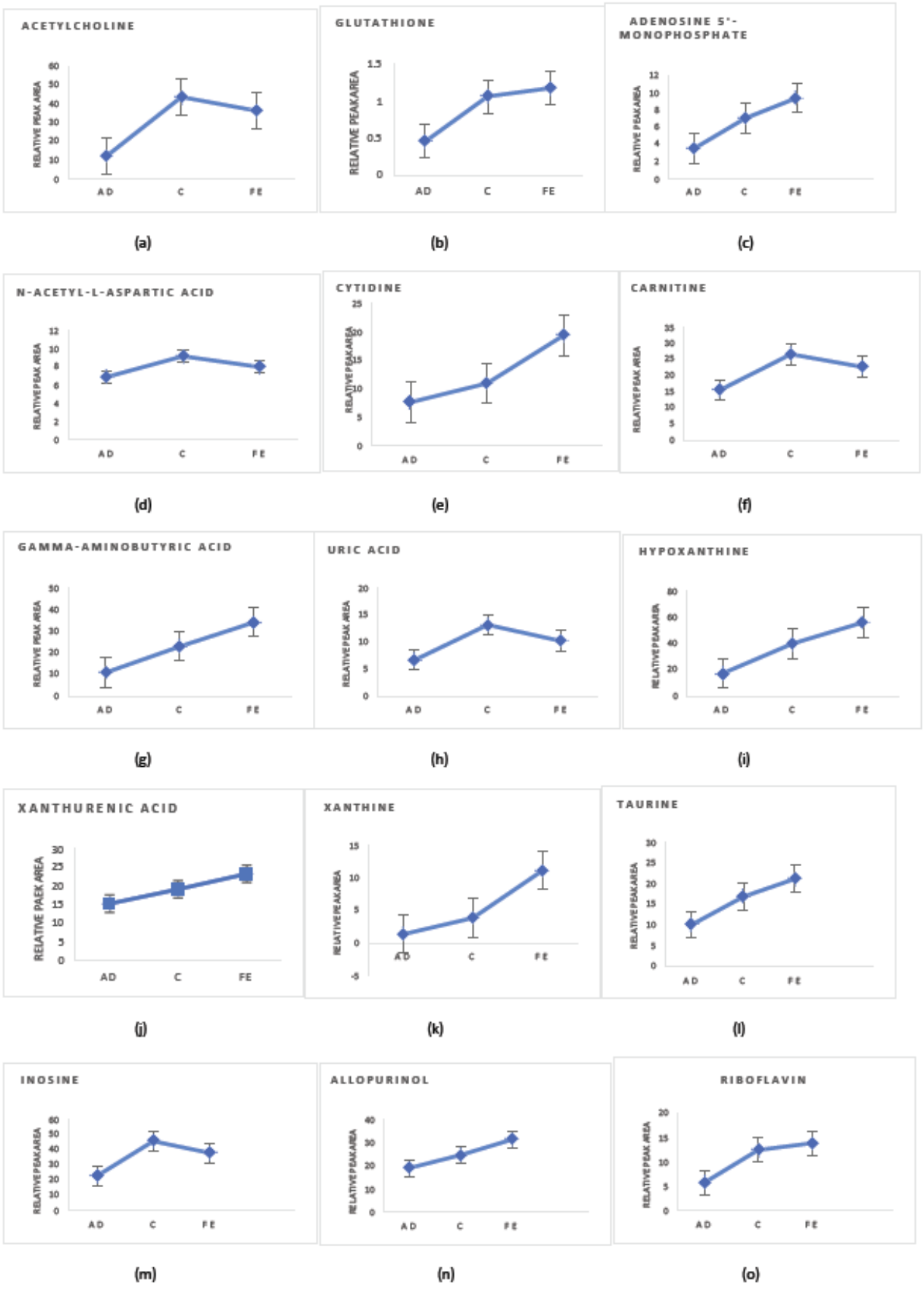


Fig. (5). Aβ-42 immunostaining (**a-f**) performed on the brain section of flies after 30 days of the exposure; a-AD fly, b- Control, c-AD + F1, d-AD + F2, e-AD + F3, f-AD + F4 (100 X) (F1 = 0.005 g/ml, F2 = 0.010 g/ml, F3 = 0.015 g/ml, F4 = 0.02 g/ml; AD = AD flies; DH = 0.1 mM Donepezil hydrochloride) a-significant at $p < 0.05$ compared to AD; b-significant at $p<0.05$ compared to AD. (**g**) Quantification of Aβ-42 aggregates from the total area of the brain using Image-J software (The values are mean of five brain sections from each treatment group). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

(Fig. **6q**; $p < 0.05$). Choline levels showed a 1.21 fold increase in AD flies compared to controls (Fig. **6r**; $p < 0.05$), with FE exposure causing a 1.09 fold reduction (Fig. **6r**; $p < 0.05$). Adenosine levels increased by 1.38 fold in AD flies compared to controls (Fig. **6s**; $p < 0.05$), but FE treatment decreased these levels by 1.17 fold (Fig. **6s**; $p < 0.05$). Histamine, mannose-6-phosphate, and MHPG levels were significantly elevated by 1.30, 1.53, and 1.68 fold, respectively, in AD flies compared to controls (Fig. **6t-v**; $p < 0.05$). FE treatment significantly reduced these levels by 1.43, 1.28, and 2.21 fold, respectively, compared to untreated AD flies (Fig. **6t-v**; $p < 0.05$).

The selected 22 metabolites (Table **2**) were analysed with the help of software, PCA was applied for the transgenic AD flies, control and AD flies exposed to FE groups. The first principal component explains 69.2 % of the variance in the data, while the second principal component, explains 15.2 % of the variance in the data (Fig. **7a**). Three different groups of flies are well-separated from each other. The separation of groups suggests that these groups have distinct profiles from each other. Similarly, PCA biplot represents the samples that are close to each other on the plot have similar metabolite profiles (Fig. **7b**). For further discrimination, PLS-DA plot was drawn suggesting that AD flies are well-separated from


ACETYLCHOLINE
RELATIVE PEAK AREA
AD
C
FE
(a)
GLUTATHIONE
RELATIVE PEAK AREA
AD
C
FE
(b)
ADENOSINE 5'-MONOPHOSPHATE
RELATIVE PEAK AREA
AD
C
FE
(c)
N-ACETYL-L-ASPARTIC ACID
RELATIVE PEAK AREA
AD
C
FE
(d)
CYTIDINE
RELATIVE PEAK AREA
AD
C
FE
(e)
CARNITINE
RELATIVE PEAK AREA
AD
C
FE
(f)
GAMMA-AMINOBUTYRIC ACID
RELATIVE PEAK AREA
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C
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(g)
URIC ACID
RELATIVE PEAK AREA
AD
C
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(h)
HYPOXANTHINE
RELATIVE PEAK AREA
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INOSINE
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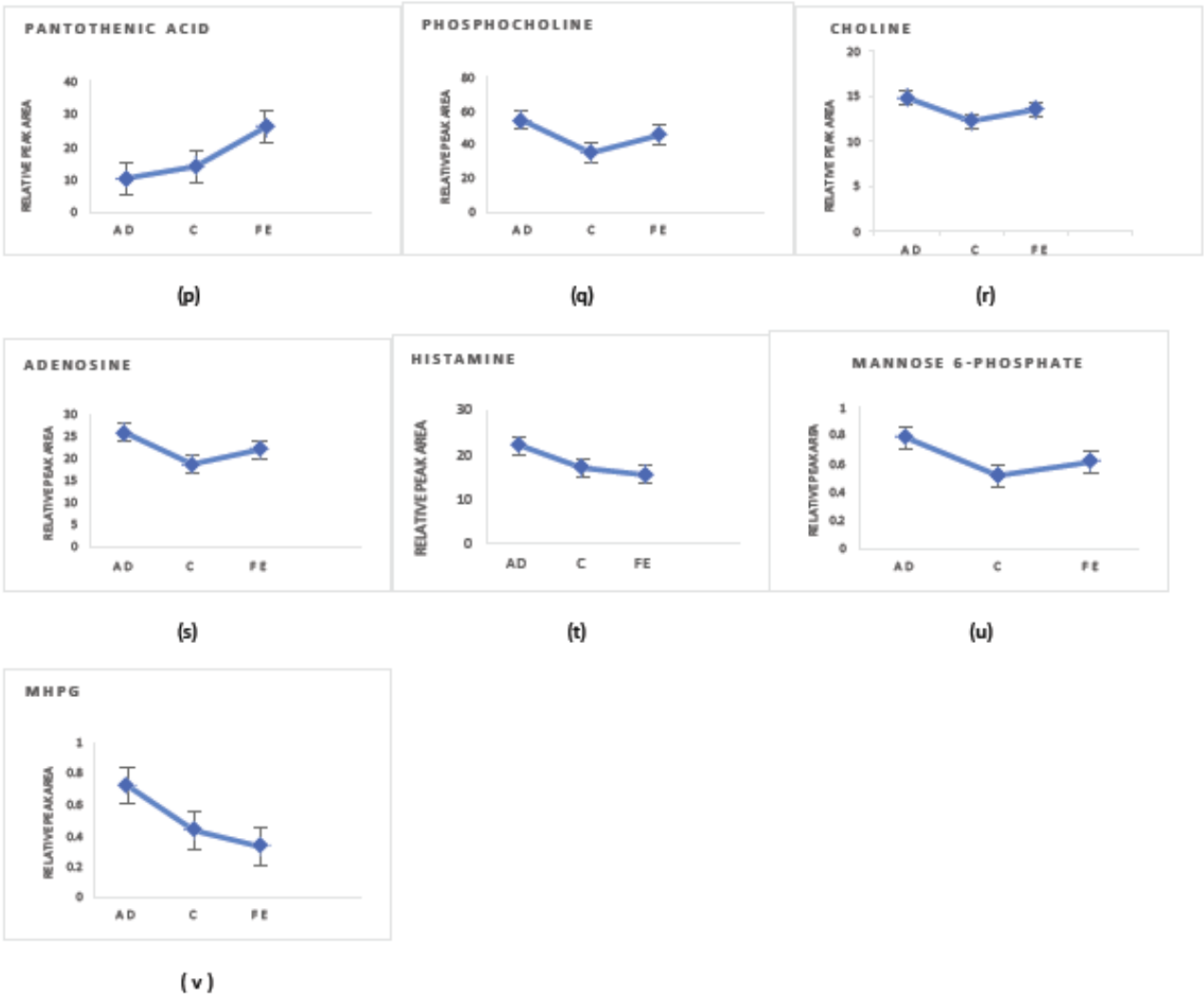

Fig. (6). (**a-v**). Metabolic profile of selected 22 compounds in the brain homogenate of AD, control and AD flies exposed to Fenugreek extract. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

control and FE exposed AD flies along component 1 (Fig. **7c**). In this model, the R^2 values are high, with 1 component at 0.87622, and increasing to 0.99811 with 3 components. The Q^2 starting at 0.74842 for 1 component and improving to 0.94205 for 3 components, indicating that the model explains large proportion of the variance in the data and well-fitted. To identify the importance of each variable to the model's predictive capability, VIP score plot was drawn (Fig. **7d**). This plot indicates that xanthine, MHPG, hypoxanthin, gama-aminobutyric acid, Adenosine-5-monophosphate, pantothenic acid and acetylcholine have the highest VIP scores, making them the most important metabolites for differentiating between the groups. To examine the abundance of metabolites in different groups heatmap combined with hierarchical clustering was done indicating blue colour showed lower relative levels, while red colour showed higher relative levels of a metabolite across different groups. Metabolites (rows close to each other in the heat map) that are grouped closely in the heat map exhibit similar patterns across the samples (Fig. **7e**). Volcano plot analysis for the selected 22 metabolites (Fig. **7f**) between transgenic AD flies *versus* control flies indicates that acetylcholine is highly downregulated and significant for the study (Fig. **7g**). Volcano plot drawn between transgenic AD flies versus exposed AD flies indicates that xanthine is highly upregulated and significant for the study (Fig. **7h**).

4. DISCUSSION

Despite present medical, psychosocial treatments, and proper care used for the effective management of AD patients, approximately 1 in 9 individuals aged 65 and older (10.9%) has Alzheimer's [27]. The substantial evidence supports that the use of plant products and plant-based extracts could prevent the progression and treatment of AD [6]. The AD flies exposed to fenugreek extract showed a reduction in free radical species formation, apoptotic markers, and Aβ-42 aggregates accumulation. The effects of fenugreek on Nrf2-mediated AD pathophysiology in rodents showed neuroprotection against neurodegeneration [28]. In our study, we found that AD flies exposed to Fenugreek leaf extract showed improvement in the loss of climbing ability and memory. It has been reported that fenugreek considerably attenuates learning and cognitive deficits in AD models [29].

Table 2. Selected metabolites used in this study with their names and compound ID.

S. No.	Metabolites Name	HMDB	PubChem	KEGG
1.	Xanthurenic acid	HMDB0000881	5699	C02470
2.	Acetylcholine	HMDB0000895	187	C01996
3.	Phosphocholine	HMDB0001565	1014	C00588
4.	Adenosine 5'-monophosphate	HMDB0000045	6083	C00020
5.	N-Acetyl-L-aspartic acid	HMDB0000812	65065	C01042
6.	Choline	HMDB0000097	305	C00114
7.	Adenosine	HMDB0000050	60961	C00212
8.	Cytidine	HMDB0000089	6175	C00475
9.	Carnitine	HMDB0000062	10917	C00487
10	Glutathione	HMDB0000125	124886	C00051
11.	Gamma-Aminobutyric acid	HMDB0000112	119	C00334
12.	Uric acid	HMDB0000289	1175	C00366
13.	Hypoxanthine	HMDB0000157	790	C00262
14.	Xanthine	HMDB0000292	1188	C00385
15.	Taurine	HMDB0000251	1123	C00245
16.	Inosine	HMDB0000195	6021	C00294
17.	Histamine	HMDB0000870	774	C00388
18.	Mannose 6-phosphate	HMDB0001078	439198	C00275
19.	MHPG	HMDB0001490	10805	C05594
20.	Allopurinol	HMDB0014581	2094	-
21.	Riboflavin	HMDB0000244	493570	C00255
22.	Pantothenic Acid	HMDB0000210	6613	C00864

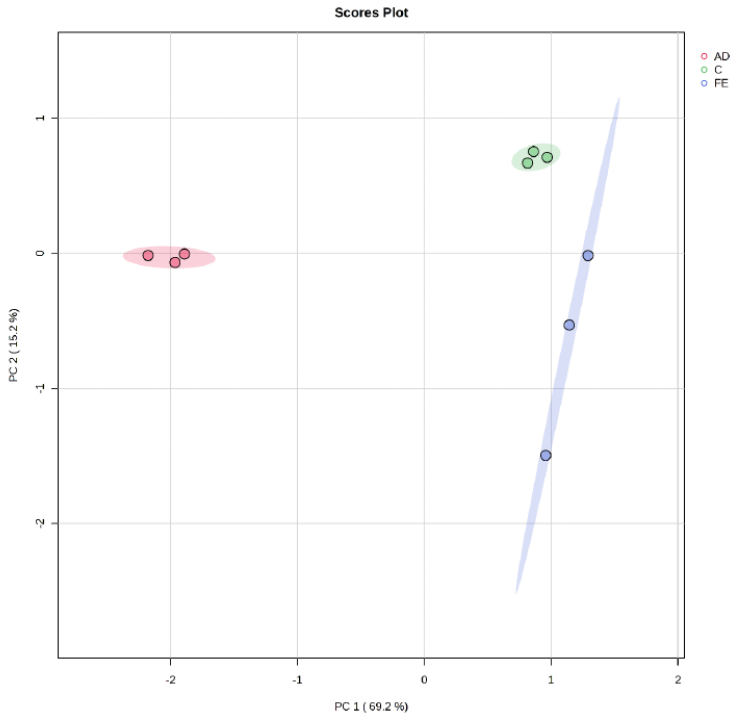

Fig 7. (a)

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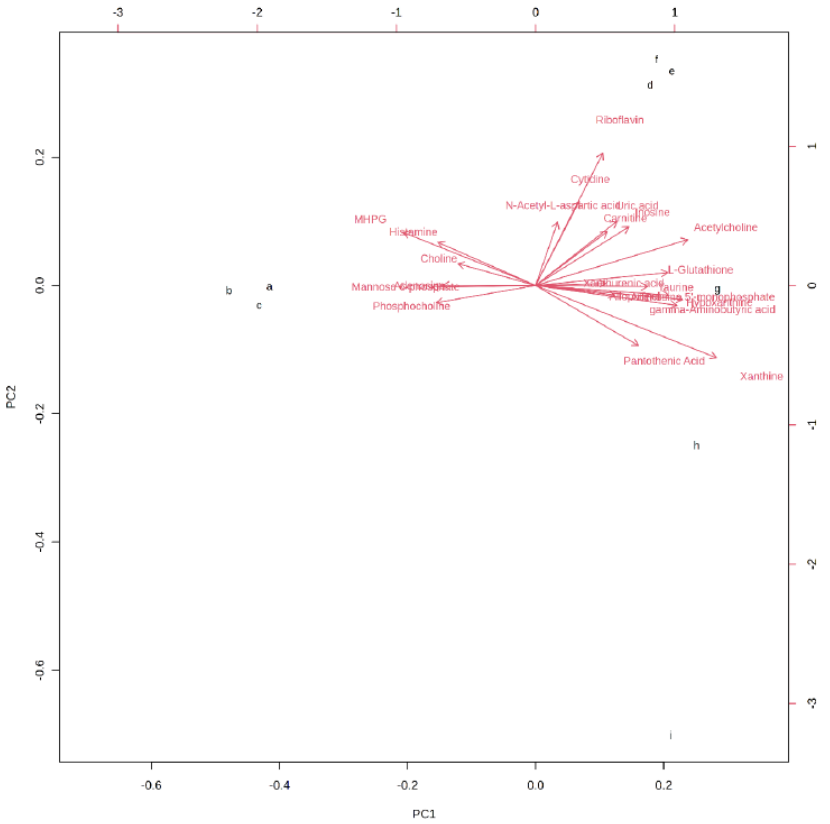

Fig 7. (b)

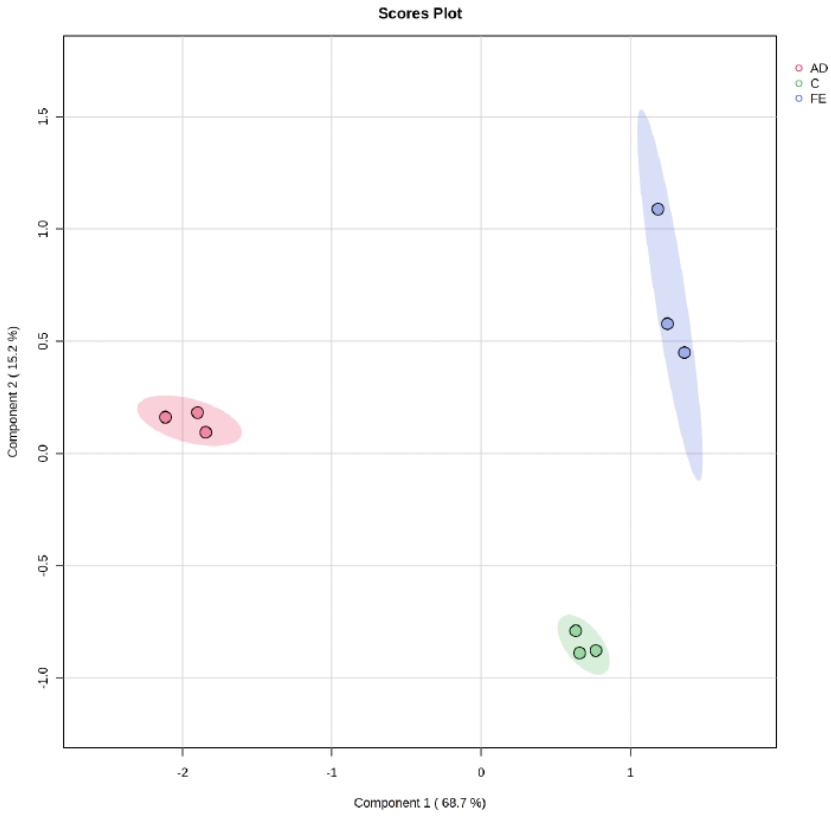

Fig 7. (c)

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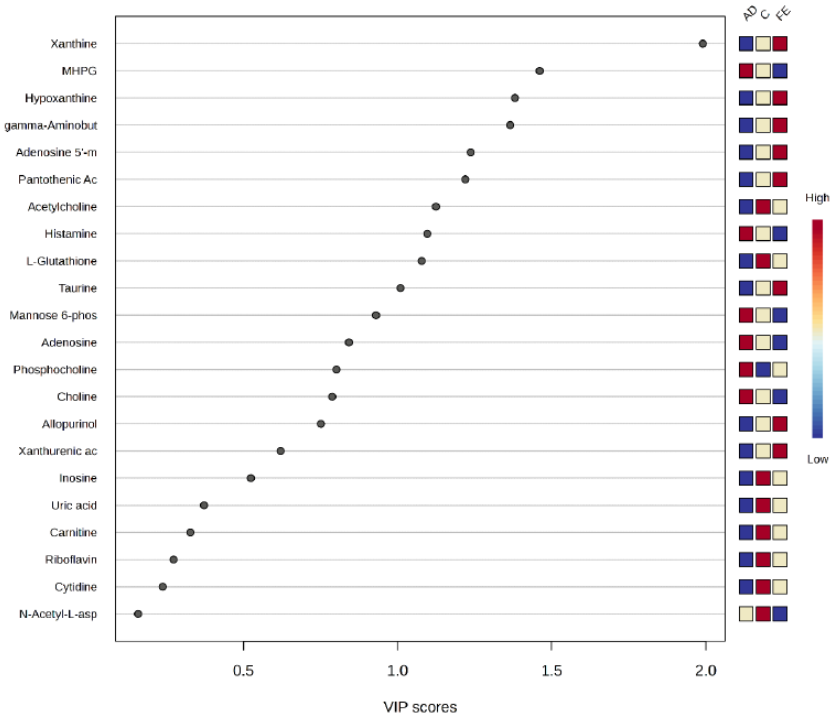

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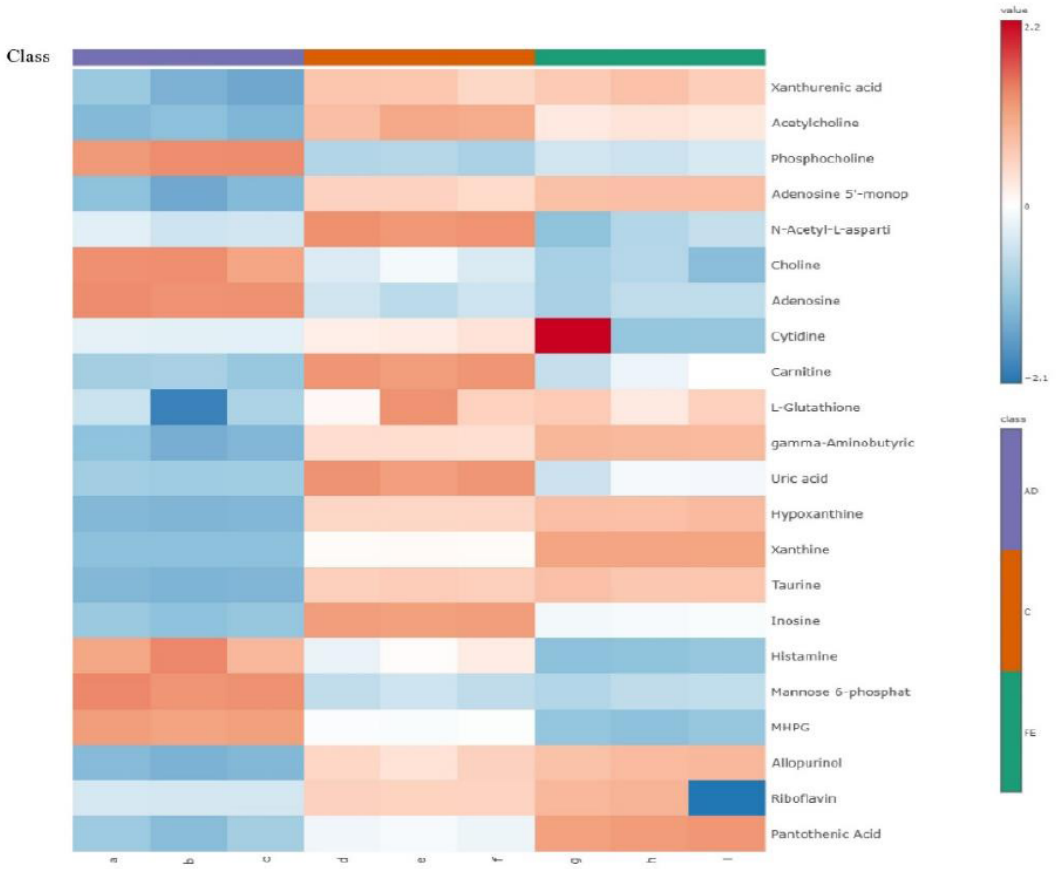

Fig 7. (e)

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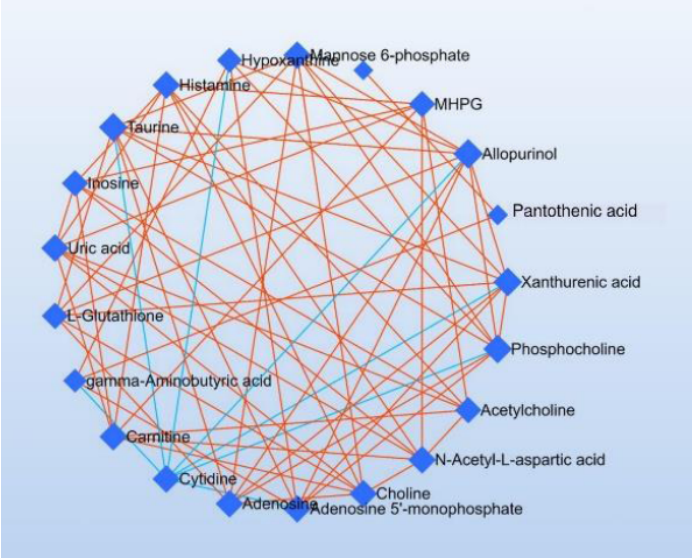

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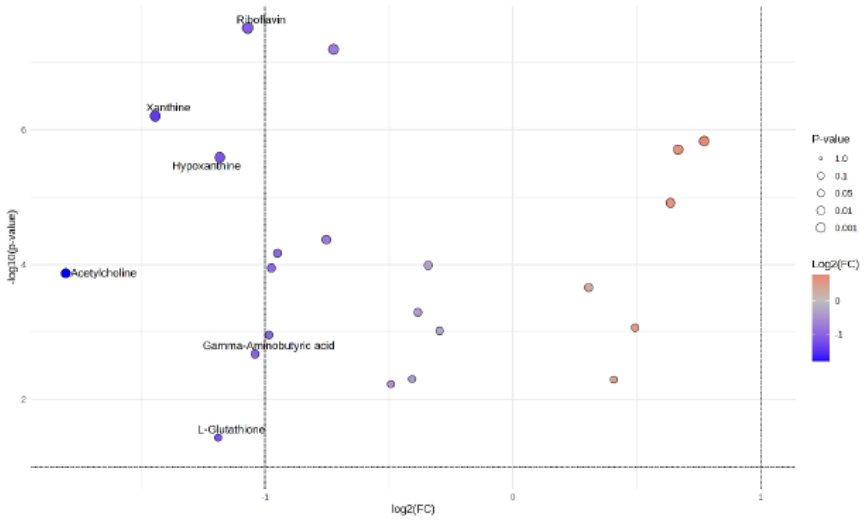

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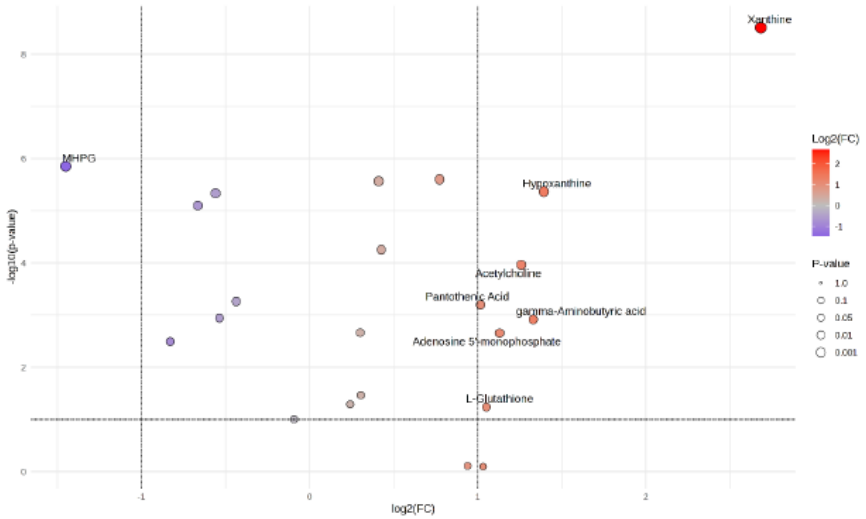

Fig 7. (h)

(Fig. 7) Contd….

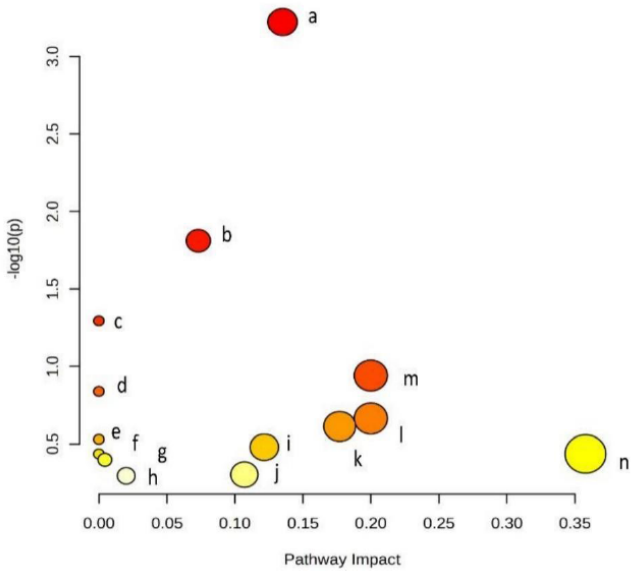

Fig 7. (i)

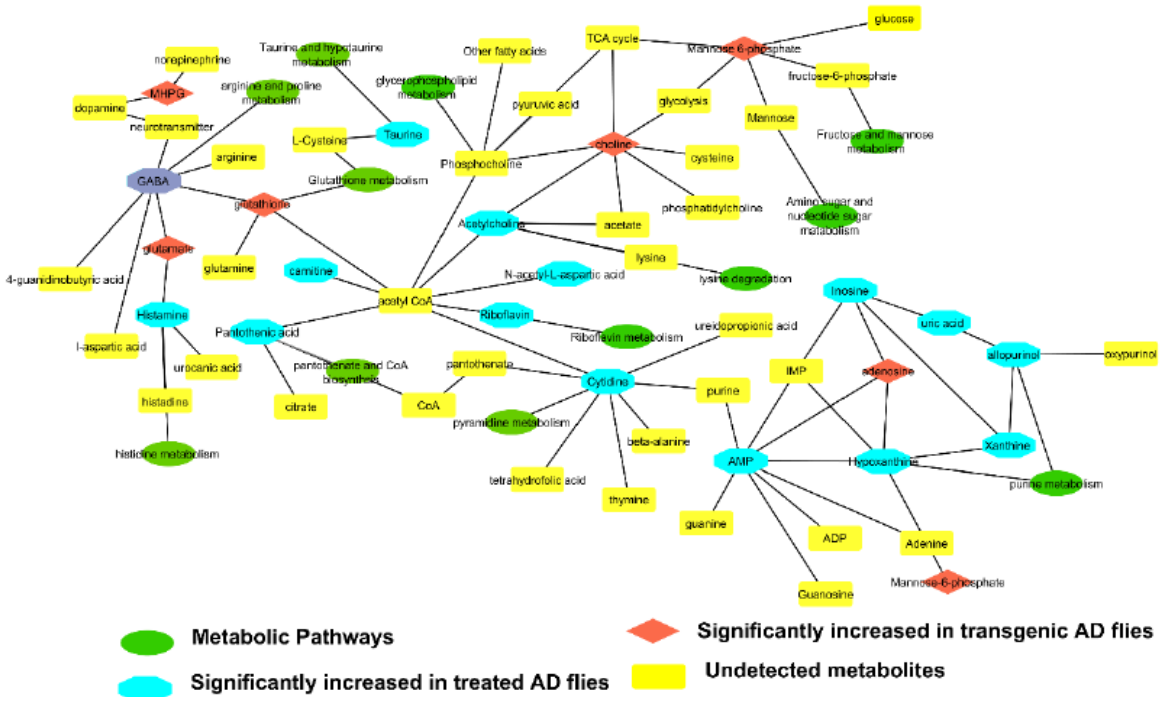

Fig 7. (j)

Fig. (7). PCA analysis of metabolites in this study: (**a**) PCA score plot of metabolites in AD flies, AD flies exposed to Fenugreek extract and control flies. (**b**) PCA biplot of metabolites in AD flies, AD flies exposed to Fenugreek extract and control flies. (**c**) PLS-DA analysis of metabolites: Score plot of metabolites in AD flies, AD flies exposed to Fenugreek extract and control flies. (**d**) VIP Score plot of metabolites in AD flies, AD flies exposed to Fenugreek extract and control flies. (**e**) Heatmap of metabolites in AD flies, AD flies exposed to Fenugreek extract and control flies. (**f**) Metabolic network analysis of metabolites used for the study. Squares represent individual metabolites, larger sizes indicating a greater influence of the metabolite on others and a higher number of associated metabolites. This implies that these metabolites are crucial in the pathogenesis of AD. (**g**) Volcano plot analysis of metabolites in AD flies versus control flies; (**h**) Volcano plot analysis of metabolites in AD flies versus flies exposed to Fenugreek extract. (**i**) Metabolic pathway involved in the metabolites consider for this study in the different group of flies a. Purine metabolism; b. Glycerophospholipid metabolism; c. Riboflavin metabolism; d. Histidine metabolism; e. Pantothene and CoA biosynthesis; f. Arginine and proline metabolism; g. Lysine degradation h. Pyramidine metabolism; i. Alanine, aspartate and glutamate metabolism; j. Amino sugar and nucleotide sugar metabolism; k. Fructose and mannose metabolism; l. Butanoate metabolism; m. Turine and hypotaurine metabolism; n.Glutathione metabolism. (**j**) Metabolic pathways associated with 22 metabolites that altered in the different groups of AD flies, control flies and AD flies exposed to Fenugreek Extract. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Biochemical and histopathological studies demonstrate that the bioactive component of fenugreek *i.e.,* diosgenin attenuates neuronal damage in the rat brain by reducing amyloid-β (1-42) aggregates deposition, oxidative stress, and neuroinflammation in the rat brain [30]. Furthermore, the study supported the protective effect of fenugreek against AD symptoms by exhibiting its acetylcholinesterase inhibitory activity and attenuating neurofibrillary tangles [31]. The results obtained for docking studies showed that acetylcholinesterase was found to form a docked complex with 2-Myristynoyl pantetheine (present in fenugreek). The active site of acetylcholinesterase comprises 2 subsites-the anionic site and the esteratic subsite. The anionic subsite binds the positively charged quaternary amine of acetylcholine, as well as other cationic substrates and inhibitors. The esteratic subsite, responsible for hydrolyzing acetylcholine into acetate and choline, features a catalytic triad consisting of three amino acids: histidine 440, serine 200, and glutamate 327. The results of docking studies on acetylcholinesterase and Aβ42 with 2-Myristynoyl pantetheine showed a favourable interaction. 2-Myristynoyl pantetheine has been reported to posses anti-Alzheimer effect [32]. It has been found that alkaloids present in fenugreek decreased Aβ accumulation *via* declining oxidative stress and advanced glycation end products in AD [33]. Pyrano[4,3-b] benzopyran-1,9-dione is known for its antioxidant, inflammatory and anti-cancerous properties [34]. Its presence can contribute to synergistic bioactivity. The other compounds present in the extract may be of pharmacological importance.

In our present study, AD flies that were fed with the diet containing different doses of fenugreek leaf extract showed increased life span in a dose dependent manner. The extended survival of exposed flies compared to AD may be due to the possible reduction in the accumulation of amyloid protein in the brain tissues and activation of the antioxidative system. Decrease in the oxidative damage and Aβ peptide aggregation might contribute to the delay of the loss of climbing behaviour. A reduction in the loss of climbing behaviour was observed in the AD flies exposed to other phytochemicals [35].

The *Drosophila* olfactory system offers a distinctive framework for investigating associative and predictive learning, with the mushroom body playing a crucial role in olfactory learning. In fruit flies, olfactory conditioning is typically explained through models of associative synaptic plasticity, which depend on correlation-based mechanisms [36]. The early stages of AD are often marked by a loss of the sense of smell, a factor that should be considered in future research employing olfactory learning and memory tests [37]. Developmental studies have indicated that both the mushroom body (MB) and the central complex are implicated in visual learning. This study further shows that the different groups of flies were studied for learning and short-term memory using the aversive phototaxis suppression assay and odour choice index. AD flies exhibited a significant decline in performance compared to the control group. However, a notable improvement in performance was observed in AD flies that were exposed to FE. Also, there is a significant alteration in locomotor behaviour of AD flies compared to control, and AD flies exposed to FE.

Several neurodegenerative diseases, including AD, have been linked to oxidative stress. The Aβ fibrils aggregation is known to induce neurotoxicity, primarily through an excess formation of reactive oxygen species (ROS), leading to processes like protein oxidation, lipid peroxidation and subsequent neuronal dysfunction [3]. Cells are protected from the ROS by an antioxidant system. This system consists of the enzymes catalase (CAT), glutathione peroxidase, and superoxide dismutase (SOD). Thus, any changes in the activity of the antioxidant enzymes may indicate the pathophysiological changes during the progression of AD [38]. The brains of AD patients and transgenic flies expressing human Aβ-42 have also been reported to have elevated ROS, PCC, TBARS, and GST activity, and reduced GSH content [38]. According to experimental studies it has been found that brain tissues of AD patients have shown increased oxidative stress during the progression of the disease [39]. Lipids, proteins and nucleic acids are modified by ROS and amyloid plaques aggregation in AD flies brains compared to controls. The oxidative membrane damage leads to the formation of malondialdehyde with several other products of oxidative stress. Superoxide dismutase leads to breakdown of superoxides to H_2O_2, and CAT converts H_2O_2 to water, while glutathione peroxides catalyzes the reduction of peroxides into less reactive molecules [40]. The ROS mediated oxidative stress results in the production of free carbonyls in the nuclei of neurons and glia cells in AD [40, 41]. Furthermore, monoamine oxidase (MAO), an enzyme located on the mitochondrial membrane, alleviates oxidative stress through the process of oxidative deamination. According to studies, AD brains have higher MAO activity in brain tissues compared to healthy brains, which is also confirmed in our study of AD flies**.** Clinical report revealed that MAO-inhibitors leads to a 20-40% reduction in cognitive decline compared to control groups [42].

Also, plants and their extract have been used as acetylcholinesterase inhibitor that contain no or minimal side effects. In our study, AD flies showed a significant higher level of AChE, leading to possible memory and behaviour impairments. Treatment with fenugreek leaf extract showed decreased AChE levels, also supported by our docking study. These results are consistent with previous reports that also conclude that fenugreek inhibits AChE [8], thus ameliorating the symptoms of dementia. The study of a randomized controlled trial including 82 AD patients recorded mild to moderate symptoms who were supplemented with fenugreek seed extract for four months demonstrated improved memory and decreased depression. Furthermore, there was a significant elevation in serum levels of total antioxidant capacity and a reduction in serum malondialdehyde (MDA) levels [43]. The AD flies exposed to fenugreek leaf extract showed decrease activity of caspase 3 and 9 in the dose-dependent manner. Exposure to the bioactive component of fenugreek has been reported to reduced the expression of Bax/Bcl-2 in the treated mice [44]. One of the studies has proved that it affects Fas (active caspase-8 and active caspase-3) and mitochondria (cytochrome c, active caspase-9 and active caspase-3) dependent apoptotic pathway [45].

Histopathological studies of the brain section of *Drosophila* expressing human Aβ-42 in the neurons showed the presence of Aβ-aggregates. The presence of these aggregates

hampers the learning and behavioural abilities of AD flies. When the AD flies exposed to the fenugreek leaf extract, showed a dose dependent reduction in the aggregation of amyloid structure in the brain section of AD flies. This may be attributed to the interaction between 2-Myristynoyl pantetheine and Aβ-42, as suggested by docking studies. Earlier, a study performed by Prema *et al,* [46] reported the reduction of amyloid biosynthesis by downregulating the expression of APP, $A\beta_{1-42}$, β and γ secretases in rats' brains treated with fenugreek seed powder. Aβ toxicity is associated with elevated levels of oxygen free radicals, such as hydrogen peroxide (H_2O_2) [5]. It has also been suggested that Aβ contributes to oxidative stress and lipid peroxidation in neuronal cultures. As a result of H_2O_2 and ROS, mitochondrial function is directly affected, and apoptotic factors are released into the cytosol [47]. Also, in mice administered *Trigonella foenum-graecum* seeds extract (TF) for 24 hours, trigonelline was detected in their cerebral cortex. 5XFAD mice cerebral cortex showed elevated levels of axonal protein and neurofilament light after TF seed extract treatment [48]. However, diosgenin (a steroidal sapogenin obtained from *Trigonella* spp) treated 5XFAD mice marked with a substantial reduction of amyloid deposits in the cerebral cortex and hippocampus [31]. The extracts of medicinal plants have been reported to reduce the insoluble amyloid beta aggregation in the brain tissue of different animal models [6]. Fenugreek (*Trigonella foenum-graecum*) extract has acquired attention as a promising plant-based therapy for AD, owing to its neuroprotective effects, including anti-inflammatory, antioxidant, and cholinesterase-inhibiting effects. Its key bioactive components, such as diosgenin, trigonelline and flavonoids, have been shown to enhance cognitive function, lower amyloid-beta levels, and influence insulin signaling pathways-bridging the connection between metabolic disorders and neurodegeneration. In comparison to other well-known botanicals like curcumin, resveratrol, *Ginkgo biloba*, *Centella asiatica, Glycyrrhiza Species, Panax ginseng,* and *Bacopa monnieri*, fenugreek presents distinctive metabolic benefits but remains underexplored in clinical settings. Despite encouraging preclinical findings, further research in mammalian models and human trials is essential to validate its therapeutic potential [6].

The GAL4/UAS expression system enables precise targeting of any vertebrate genes to specific tissues and cells within *Drosophila*, facilitating controlled and specific gene expression patterns [49]. By introducing transgenes encoding human APP protein (HAPP), human β-secretase protein (hBACE), and *Drosophila* γ-secretase presenilin (dPsn) into *Drosophila*, researchers induced a condition where the cleavage of *Drosophila* APP1 (dAPP1) could initiate, resulting in the generation human Aβ peptide [45]. This process led to age-dependent behavioral impairments and neurodegeneration. The transgenic *Drosophila* expressing Aβ42 peptide provided a model for studying the toxicity and associated neurodegenerative mechanisms of Aβ peptide. The similarity in the Aβ generated pathogenesis of AD in *Drosophila* and clinical symptoms of AD in humans makes *Drosophila* subsequently useful for the study of Aβ neurotoxicity [50]. The simplicity of genetic manipulation and the ability to conduct experiments efficiently enable the utilization of large-scale forward genetic screens in *Drosophila* models, facilitating the rapid identification of modifiers associated with diseases [51].

In this study, changes in the metabolic profile of *Drosophila* associated with AD was also studied. Among the number of metabolites, we studied 22 metabolites in AD, control and AD flies exposed to FE. Metabolomics helps us in understanding of the biochemical processes involved in diseases and may offer novel biomarkers for AD. These distinct metabolites play roles in energy metabolism, neurotransmission, and cognitive function. The metabolites related with purine, glycerophospholipid, riboflavin, Amino sugar and nucleotide sugar, fructose and mannose, histidine, pantothene and coA biosynthesis, arginine and proline, lysine, pyramidine, aspartate, glutamate, and alanine, butanoate, taurine and hypotaurine, and glutathione metabolism (Fig 7 i & j). Among the number of metabolites, some metabolites were significantly decreased in the transgenic AD flies. These include uric acid, hypoxanthine, xanthine, taurine, inosine, and allopurinol belong to the purine metabolic pathway. In the earlier findings, uric acid has shown a protective role against the adverse effects of Aβ aggregates. Numerous studies have indicated that serum uric acid (SUA) levels are lower or tend to decrease in patients with AD [52]. During the early stages of AD-related pathology hypoxanthine levels were reduced specifically in the frontal cortex, and the alterations in the levels of the acetylcholinesterase and butyrylcholinesterase activities in the brain tissue has also found [53]. Among its many functions, taurine is involved in neurotransmission, neuroregulation, osmoregulation, calcium influx control, and controlling cell excitability [54]. Another metabolite, which was found to be reduced in the AD flies, was inosine, which is formed by the breakdown of adenosine. Earlier studies have indicated its antioxidative and acetylcholinesterase inhibitor effect [55]. AD patients reportedly showed an increase in histamine serum levels, which contributed to cognitive and memory impairments in the AD patients. Likewise, our AD group flies showed a significant increment in the histamine affecting histidine metabolic pathway. Numerous studies have documented alterations in the biogenesis and metabolism of carnitine in individuals with AD [56]. Allopurinol showed a decrease in AD flies compared to control and treated flies. Animal studies have shown that allopurinol, a xanthine oxidase inhibitor, can decrease oxidative stress [57]. These findings led to the hypothesis that allopurinol may also inhibit xanthine oxidase and potentially lower the risk of AD [57]. Among the reduced metabolites, cytidine, which is important for the formation of phosphatide in the brain, *via* the Kennedy pathway was reported to enhance the production and release of neurotrophic APP, which promotes neuron growth and health, potentially providing therapeutic advantages for neurodegenerative diseases [58]. Metabolite like gama-amino butyric acid was also reported to decline in AD flies, compared to exposed AD flies and control flies. Disruption of GABAergic function can contribute to neuroinflammation and has been associated with cognitive decline and memory loss [59]. The decreased levels of carnitine have been reported in AD patients [56]. Also, Neuronal glutathione depletion was marked in the brains of AD mice, while no such alteration was observed in the brains of control subjects [60]. Similarly, a decrease in the level of acetylcholine was found in AD

patients. It facilitates the communication between neurons in regions like the hippocampus and cerebral cortex, which are heavily involved in memory processing and higher cognitive functions [61]. AD flies in this study exhibit significantly reduced levels of N-Acetyl-L-aspartic acid. Lower N-Acetyl-L-aspartic acid levels in AD patients have been considered as a biomarker of neuronal dysfunction and loss, as it is primarily found in neurons [62]. Likewise, cerebral deficiency of pantothenic acid was found in AD, which is a precursor of acetyl-CoA, and is required for the synthesis of acetylcholine. The brains of AD patients have reduced levels of pantothenic acid, confirming our observations of major alterations in pantothenic acid in AD flies compared to treated, and control flies. Earlier, a study revealed that increased riboflavin consumption was linked to a reduced likelihood of cognitive decline among adults in the United States [63]. Alterations in riboflavin metabolism contribute not only to cognitive function, but also acts as an antioxidant and anti-inflammatory agent [64]. AD patients exhibit increased levels of choline metabolites along with disrupted glycerophospholipid metabolism [65]. Apart from acetylcholinergic system, the disturbed histaminergic system also contributes cognitive deficits in AD patients. Notably, histamine levels in the blood are higher in early-onset AD compared to late-onset cases [66]. Also, the finding of our study reveals the upregulated level of histamine in the AD flies relative to control flies. Moreover, metabolites like MHPG, mannose-6-phosphate, adenosine, xanthurenic acid, choline and phosphocholine were significantly increased in AD flies group. It has been found that there is a alteration in the expression of human mannose-6-phosphate receptor in a mouse fibroblast-like cell line in sporadic AD [67]. Studies suggest that overexpression of the IGF-II/M6P receptor has been linked to elevated levels and modified amyloid precursor protein processing, resulting in the production of β-amyloid peptide [68]. Adenosine impacts key brain functions, such as sleep, arousal, cognition, memory, as well as neuronal injury and degeneration [53]. According to the research, early AD is caused by enhanced synaptic ATP release and an increase in the density and activity of ecto-5′-nucleotidase (CD73), which leads to increased adenosine production, which activates A2A receptors specifically [69]. Notably, in our study, adenosine was found to be increased in the AD flies group compared to exposed AD and control flies. Furthermore, hyperactivity of the noradrenergic system was observed in AD condition, which leads to increased level of cerebrospinal fluid and 3-Methoxy-4-hydroxyphenylglycol (MHPG) (metabolite of the monoamines adrenaline and noradrenaline) in AD patients [70]. Similarly, we found a significant increment in the MHPG level in AD flies compared to treated and control flies. A metabolite of the kynurenine pathway, xanthurenic acid, may not only be useful as a marker for the preclinical stage of Alzheimer's disease, but also as a target for anti-amyloid drugs [71]. Studies indicate that reduced levels of xanthurenic acid in the blood were associated with preclinical Alzheimer's disease risk factors, including oxidative stress, Aβ42 accumulation, and increased astrocyte proliferation [71]. This research demonstrates that AD is linked to widespread metabolic alterations in the brain. Several metabolites, such as GABA, phosphorylcholine, and MHPG, have also been previously detected in the cerebrospinal fluid (CSF) of AD patients. However, the identification and alteration of distinct metabolites across various brain regions suggest their possible role as biomarkers for AD.

CONCLUSION

This study demonstrated the neuroprotective effect of fenugreek extract *via* its abilities to improve the behavioural pattern, enzyme activities, cognitive impairment, oxidative stress and histopathological changes in the brain tissue. A phytochemical-based bioactive agent might be responsible for this protective effect revealed in the GCMS analysis, having 2-Myristynoyl pantetheine with maximum peak area in the fenugreek extract (Table **S1**). This compound fits into the pocket of acetylcholinesterase and Aβ-42 protein aggregates as revealed in the docking result. Additionally, fenugreek extract suppresses acetylcholinesterase enzyme activity in AD flies. Fenugreek extract reduced amyloid protein loads in the head sections of AD flies. Further, LCMS analysis of heads of AD flies, AD flies exposed to fenugreek extract, and control flies showed the altered level of metabolites involved in the diseased pathophysiology. These results indicate that fenugreek extract could be a potential drug among medicinal plants for the treatment of AD. Herbal products may demonstrate encouraging effects in *in vitro* experiments or in simpler model organisms such as fruit flies; however, these results often fail to translate effectively to more complex mammalian systems like rodents or humans, primarily due to significant differences in physiology and brain structure. Invertebrate models like *Drosophila*, with their relatively simple neural architecture and limited immune response, cannot fully mimic the complexity of AD. Additionally, the poor bioavailability of many plant-based formulations can limit their therapeutic effectiveness. Therefore, extensive testing in mammalian models, particularly rodents, followed by well-designed clinical trials in humans, is essential. Such studies are necessary to clarify the underlying mechanisms and to support the development of these interventions into reliable, targeted therapies. Evidence supported that fenugreek provides promising options as ingredients of nutraceutical agents from various animal models of AD research. Streptozotocin (STZ)-induced rat model after fenugreek treatment showed improvement in cognition and behavioural activities. Also, fenugreek improves biochemical profile and inhibits acetylcholinesterase activity of Scopolamine-induced cognitive impairment in the rodent model. $AlCl_3$ causes oxidative stress and amyloid formation, often used to model AD-like traits, appeared to be improved by the supplement of fenugreek. Furthermore, active constituents of fenugreek alleviate amyloid formation in the β-amyloid induced rodent model.

STUDY LIMITATIONS

While this study has certain limitations, the findings suggest that fenugreek leaf extract may serve as a promising natural candidate for modulating AD-like pathophysiology and related metabolic alterations, thereby warranting further investigation in more complex biological systems. Nonetheless, critical aspects such as optimal dosage, absorption, and metabolic processing of FE in higher organisms remain poorly defined. Therefore, the neuroprotective effects observed in *Drosophila* should be validated in mammalian

models and eventually in human trials to establish their translational potential.

FUTURE DIRECTIONS

Bioactive compounds of fenugreek, like Trigonelline, galactomannan, diosgenin, flavonoids, sapogenins and others can be extracted by using these major emerging technologies, including ultrasound, microwave, cold plasma, and integrated technological approaches employed in the development of fenugreek-derived products. Utilizing omics-based approaches, including metabolomics and transcriptomics, in upcoming research may help uncover the molecular mechanisms and specific targets modulated by fenugreek, facilitating the advancement of fenugreek-based therapeutic or adjunct strategies for AD. The isolated compounds of fenugreek could be studied on mice/rat model of AD for a better understanding of the therapeutic potential.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Study conception and design were presented by: YHS; Data collection was done by: HV, MI, MS; Data Analysis or Interpretation was performed by: HV, MI, MS, SJ; Methodology was presented by: HV, YHS, MI, MS, IS, KJ, JF, R investigation was conducted by: HV; draft manuscript was presented by: HV, YHS, MS, MI . All authors reviewed the results and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

AD = Alzheimer's Disease
AChE = Acetylcholinesterase
APS = Aversive Phototaxis Suppression
APP-L = APP-like Protein
BCIP = Bromo-4-chloro-3-indolyl Phosphate
BHT = Butylhydroxy Toluene
CAT = Catalase
DAM = *Drosophila* Activity Monitor
DH = Donepezil Hydrochloride
FLE = Fenugreek Leaf Extract
GABA = Gamma-aminobutyric Acid
GCMS = Gas Chromatography Mass Spectrometry
GSH = Glutathione
GST = Glutathione-S-transferase
HESI = Heated Electrospray Ionization
HAPP = Human APP Protein
hBACE = Human β-secretase Protein
MAO = Monoamine Oxidase
MB = Mushroom Body
MDA = Malondialdehyde
MHPG = 3-Methoxy-4-hydroxyphenylglycol
NBT = Nitro Blue Tetrazolium Chloride
OCI = Odour Choice Index
OPA = Orthophosphoric Acid
PCC = Protein Carbonyl Content
PCA = Principal Component Analysis
PLS-DA = Partial Least Squares Discriminant Analysis
ROS = Reactive Oxygen Species
SOD = Superoxide Dismutase
SUA = Serum Uric Acid
TF = Trigonella Foenum-graecum Seeds Extract
TBA = Thiobarbituric Acid
TCA = Trichloroacetic Acid

ETHIC APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not Applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study can be obtained from the corresponding author upon reasonable request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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