Evaluation of *In Vitro* and *In Silico* Anti-Alzheimer potential of ethanolic extract of *Nyctanthes arbortristis* flowers.

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ABSTRACT:

Alzheimer's disease (AD) is a primary degenerative disease of the central nervous system. Increased level of the enzyme acetylcholinesterase (AChE) plays a key role in the hydrolysis of the neurotransmitter Acetylcholine which worsens the condition of cognitive dysfunction. Among the pathologic hypotheses of Alzheimer's disease, cholinergic deficit and oxidative stress have been implicated as two major hallmarks. Hence, inhibition of cholinesterase and oxidation are the two important strategies in the development of a drug for AD. The *in vitro* antioxidant and anticholinesterase activities of ethanolic extracts of *Nyctanthes arbortristis* Linn. were evaluated using enzymatic and chemical methods. Antioxidant potentials were evaluated by the DPPH assay and lipid peroxidation method. Anticholinesterase activity was measured by the modified Ellman method. The molecular docking study was carried out using the molecular docking tool UCSF Chimera tool to explain the interaction of the major chemical constituents with the enzymes. In antioxidant *in vitro* evaluation, IC_{50} values of the ethanolic extract of *Nyctanthes arbortristis* were found to be 64.96 µg/ml and 79.27 µg/ml for DPPH and lipid peroxidation respectively; The ethanolic extract of *Nyctanthes arbortristis* showed potent anticholinesterase effects with an IC_{50} value of 28.54 µg/ml. The docking experiments showed that Quercetin has the highest binding score among the six major identified compounds. Quercetin also mimics the standard drug galantamine that shows the same H-bond interaction with THR62 amino acid residue resulting in more potent inhibitory activity than galantamine. The ethanolic extract of flowers of *Nyctanthes arbortristis* showed promising antioxidant and anticholinesterase activity and can be used to treat Alzheimer's disease.

Keywords: *Nyctanthes arbortristis*, anticholinesterase, Alzheimer's disease, antioxidant.

1. INTRODUCTION

The oxidation of cell lipids and DNA damage are caused by free radicals such as superoxide anion, hydroxyl and peroxyl radicals, which are generated in biological systems and foods.

These radicals can lead to major diseases like cancer, coronary arteriosclerosis, and diabetes mellitus.[1] Antioxidants included in food may be useful in preventing oxidative damage. Since some synthetic antioxidants have hazardous side effects, many researchers have concentrated on studying edible and medicinal plants to find natural antioxidants. Natural antioxidants may also play a significant role in preserving human health.[2,3] Antioxidants are crucial to slowing the ageing process since oxidative stress has been shown to affect both intrinsic and skin ageing.[4,5]

It is believed that the neurotransmitter acetylcholine, which is hydrolyzed by the enzyme acetylcholinesterase (AChE), contributes to the pathophysiology of Alzheimer's disease.[6] One of the most important approaches for treating this disease involves enhancing acetylcholine levels in the brain using AChE inhibitors.

The anti-cholinesterase effect of plant extracts and medications has been demonstrated in several research. According to some reports, a few herbal extracts may affect the central nervous system and improve learning and memory. Natural products are one of the best sources for new anticholinesterase medications.[7] *Nyctanthes arbortristis* (*N. arbortristis*) is a valuable medicinal plant which belongs to the family Oleaceae. An apocarotenoid called as Crocin (I) is the colouring principle present both in saffron and in the calyx of *N. arbortristis*.[8] As a result, the calyx of *Nyctanthes arbortristis* can be used extremely effectively in place of the very expensive stigma of Crocus sativus. Flowers were investigated for phytochemical and pharmacological studies since they are widely available in India. Studies on crocin have shown that it possesses anti-inflammatory, analgesic, and antioxidant properties.[9] Dried flowers showed sedative activity, anti-inflammatory and analgesic activity. In a recent study, attempt had taken to evaluate the antioxidant and anticholinesterase activity of ethanolic extract of flowers of *Nyctanthes arbortristis.*

2. MATERIALS AND METHODS

2.1 Chemicals:

Thiobarbituric acid , trichloroacetic acid , and 2,2 Diphenyl-1-picryl Hydrazyl Hydrate (DPPH) were purchased from Himedia, Mumbai. Quercetin, 2-deoxy 2 ribose, xanthine oxidase, pyrocatechol and hypoxanthine were bought from SRL Chemical, Mumbai. All other chemicals utilized in the investigation were of analytical quality and obtained from local suppliers.

2.2 Plant material

The fresh flowers of *Nyctanthes arbortristis* were collected from the local area in the early morning of June and July. The flowers were cleaned and dried under shadow. Dried flowers were used for research work. Crude drug was authenticated at the Department of Botany, Guru Nanak Khalsa College, Matunga, Mumbai, through morphological features. (Voucher Specimens numbers: abn p 0651018) (*Nyctanthes arbortristis*).

2.3 Extraction

Cold extraction technique was used for the extraction of flowers of *Nyctanthes arbortristis*. The cleaned flowers were then shade dried and extracted with ethanol by macerating in dark for 6-8 hours at room temperature. By filtering off the marc, the ethanolic extract of *Nyctanthes arbortristis* flowers (EENA) was made clear. It was then concentrated in a Rotary flash evaporator. After that, the concentrated extract was dried out in a vacuum oven at a temperature not higher than 50°C.

2.4 Qualitative Phytochemical Analysis

The methanol extract was tested for the presence of secondary metabolites. Standard procedures were used for identifying the phytoconstituents.

2.5 Animals

Wistar albino rats of 150- 200 g were used for the study. The inbred colonies of rats were purchased from Bharat Serum, Mumbai. They were acclimatized to controlled conditions of temperature (23±2ºC), humidity (50±5%) and 12 h light-dark cycles. The protocol was sanctioned by CPCSEA, New Delhi (proposal number: GIP/IAEC/2018/10/2).

2.6 *In vitro* antioxidant activity

2.6.1 DPPH radical scavenging assay[10]

In the presence of a DPPH stable radical, the potential of extracts to donate hydrogen was investigated. 2.5 ml of sample solutions at various concentrations were mixed with one millilitre of a 0.3 mM DPPH ethanol solution and allowed to react at room temperature. The absorbance readings were measured at 517 nm after 30 minutes. As a blank, ethanol (1.0 ml) and plant extract solution (2.5 ml) were used. As a negative control, DPPH solution (1.0 ml, 0.3 mM) and ethanol (2.5 ml) were used. Standard (Ascorbic acid) solutions were used as the positive controls.

2.6.2 Assay of lipid peroxidation method [11]

Thiobarbituric acid (TBA) reaction technique was used to calculate the amount of lipid peroxidation produced by the Fe2+ ascorbate system in rat liver homogenate.

The reaction mixture consisted of rat liver homogenate 0.1 ml (25% w/v) in Tris –HCL buffer (20 mM, pH 7.0), potassium chloride (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbate (0.06 mM), and various concentrations of the EENA in a final volume of 0.5 ml. At 37 ºC, the reaction mixture was incubated for one hour. 0.4 ml of the sample was taken after the incubation period and treated with 0.2 ml of sodium dodecyl sulphate (SDS) (8.1%), 1.5 ml of TBA (0.8%), and 1.5 ml of glacial acetic acid (20%, pH 3.5).

The Total volume was made upto 4 ml of distilled water and then kept in a water bath at 95-100 ^{o}C for 1 h. after cooling, 1.0 ml of distilled water and 0.5 ml of n- butanol and pyridine mixture (15: 1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000 g for 10 min. the organic layer was removed and its absorbance at 532 nm was measured. By comparing the OD of the treatments with that of the control, the inhibition of lipid peroxidation was established. Ascorbic acid served as the standard for comparison.

Calculation of 50% inhibitory concentration (IC_{50})

The percentage scavenging activities at five different concentrations of the extract were used to determine the concentration (µg/ ml) of the extract needed to scavenge 50% of the radicals.

Using the formula, percentage inhibition (I%) was determined.

$$I\% = [Ac-As/Ac]*100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

2.7 *In vitro* acetylcholinesterase inhibition assay

The AChE inhibition activities of selected extracts were determined by slightly modifying Ellman's spectrophotometric method.[12] Acetylthiocholine iodide was used as substrate and 5,5 - Dithiobis [2- nitrobenzoic acid] was used for the measurement of cholinesterase activity and rat heart homogenate was used as the source of acetylcholinesterase enzyme. `

2.7.1 Preparation of the enzyme

A Wistar strain Rat was sacrificed by cervical dislocation, and its heart was promptly removed and homogenised in 0.1mM sodium phosphate buffer (pH 7.0) under cold conditions. The homogenate was stored until use at -80°C.

2.7.2 Assay procedure

Different extract concentrations in 2.6 ml of 0.1mM sodium phosphate buffer (pH 8.0) were added to 100 µl of DTNB (0.75 mM) and 5 µl of cardiac homogenate (Crude enzyme) and incubated at 25°C for 5 minutes. The reaction was then initiated by the addition of 20 µl of acetylthiocholine. The hydrolysis of acetylthiocholine was monitored by the formation of yellow 2- nitro- 5- sulfidobenzene carboxylate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine for 10 min, at a wavelength of 412 nm. The percentage Inhibition of cholinesterase activity was calculated using the following formula:

$$\%\ inhibition = C - S / C \times 100$$

Where C is the absorbance of the control reaction (containing all reagents except the test compound), and S is the absorbance of the test compound. Galantamine was used as positive control and all tests were carried out in triplicate.

2.8 Statistical analysis

Tests were carried out in triplicate for 3- 5 separate experiments. The amount of extract needed to inhibit free radicals' concentration by 50%, IC_{50} was graphically determined by a linear regression method. Results were expressed as graphically mean ± SD.

2.9 Molecular Docking Studies

Molecular docking investigation of the plant's active constituents *Nyctanthes arbortristis* was carried out to learn about the potential mode of action on Intel Core i5 11th Generation CPU, with 8 GB DDR2 RAM and SSD512 system. All molecules were drawn using the ChemBioDraw 14.0. Employing the UCSF Chimera tool, all the drawn compounds were prepared for docking studies. Further, the ligands were desalted, and tautomers were generated. Specified chiralities were retained and stereoisomers were generated per ligand.[13] The generated ligand molecules' 2D structure was transformed into a 3D structure that reduced energy and was then used for docking. The X-ray crystal structures of the protein validated as anti-Alzheimer's target were retrieved from protein data bank (PDB). Choline esterase (**PDB ID: 1EVE**), was imported into the protein preparation wizard tool of BIOVIA Discovery Studio. In the first step, imported protein is checked for any missing residues or loops. Then, hydrogens are added, bond-order corrected, and water molecules removed. In the next step, added hydrogens are optimized, and protein is put for restrained energy minimization using CGenFF forcefield.[13]

The binding site was verified by redocking the ligand. Molecular docking calculations of all the test compounds were carried out with Auto dock vina.[14] The conformation with the lowest binding free energy was used for analysis. Docked images of the compounds with highest binding energy and interaction i.e., Quercetin are shown in Fig. 3 to 6. Interaction in the form of binding energy of all the docked compounds including the standard drugs is presented in Table 1.

3. RESULTS

3.1 Phytochemical screening

Phytochemical screening of the plant extract revealed the presence of flavonoids, terpenoids, saponins, steroids and glycosides.

3.2 In vitro antioxidant activity

3.2.1 DPPH radical scavenging assay

The radical scavenging activity of EENA was determined from the reduction in the optical absorbance at 517 nm due to the scavenging of stable DPPH free radicals. A positive DPPH test suggests that the EENA is a potential free radical scavenger. EENA showed strong

scavenging activity (fig.1). In this assay, the IC_{50} value was found to be 64.96 µg/ml for EENA while for standard ascorbic acid, the IC_{50} was found to be 14.55 µg/ml.

3.2.2 Lipid peroxidation

EENA showed potential inhibitory effect against lipid peroxidation which is induced by Fe^{2+}- ascorbate system in rat liver homogenate. Malondialdehyde (MDA) is a toxic aldehydic end product of the reaction of lipid peroxidation with thiobarbituric acid. MDA levels were significantly reduced in presence of the EENA (fig. 1) and the IC_{50} value was found to be 79.27µg/ml for EENA while for standard ascorbic acid, the IC_{50} was found to be 19.05 µg/ml.

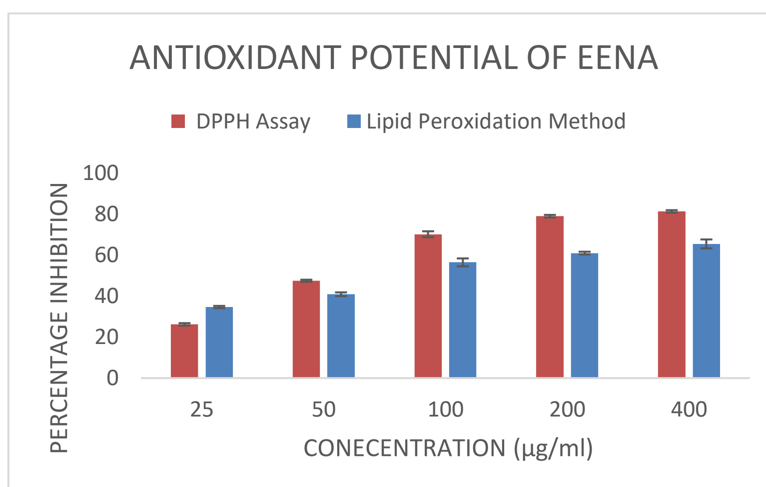


Fig. 1: Antioxidant potential of the EENA in the DPPH assay and lipid peroxidation method.

3.3 Anticholinesterase activity

In the present study, *Nyctanthes arbortristis* showed dose-dependent inhibition of AChE and the results are presented in fig. 2. EENA was found to inhibit AChE to an extent of 82.28% at 400 µg/ml concentration. In this anticholinesterase activity, the standard galantamine showed IC_{50} of 5.56 µg/ml while EENA showed moderate levels of cholinesterase inhibition also with IC_{50} of 28.54 µg/ml which is relevant for its potential for treating AD.

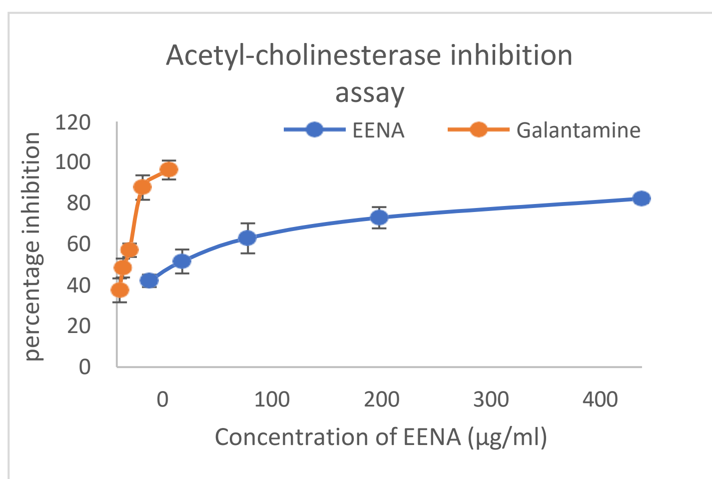


Fig. 2: Anticholinesterase activity of the EENA and Galantamine.

3.4 Molecular Docking results

The docking studies revealed that Quercetin and Kaemferol of the API's from plant extract molecules have better binding energy and several molecular interactions towards the target than the standard, and were responsible for the observed affinity. The best docking energy model and most possible interaction mode of the active compound Quercetin with protein 1EVE is shown in Fig. 3,4,5 and 6. It was observed that the compound Quercetin mainly interacts with the target enzyme by showing hydrogen bonding interaction with THR62, TYR63 and ASP93 residue, mimicking a co-crystallized ligand that shows interaction with TYR96 amino acid residue and also copies the hydrophobic interactions with ASN65, GLY32, ASN59 and PRO64 amino acid residues (see Fig. 3). Binding affinity value of the docked target compounds were found to be in the range -4.8 to -7.0 kcal mol-1. The results revealed that the benzopyran ring attached with O-H in quercetin shows strong hydrogen bonding interaction with the amino acid residues of the A chain of protein 1EVE at THR62, TYR63 and ASP93 amino acid residue, which clearly advocates its better anti-Alzheimer's efficacy. Quercetin also mimics standard drug galantamine that shows the same H-bond interaction with THR62 amino acid residue. Quercetin also has other similar interactions to standard drug with respect to its Pi-Pi T shaped interaction with TRP58 amino acid residue. From these results, it can be inferred that compound probably shows its anti-Alzheimer's activity in a similar way as that of Galantamine.

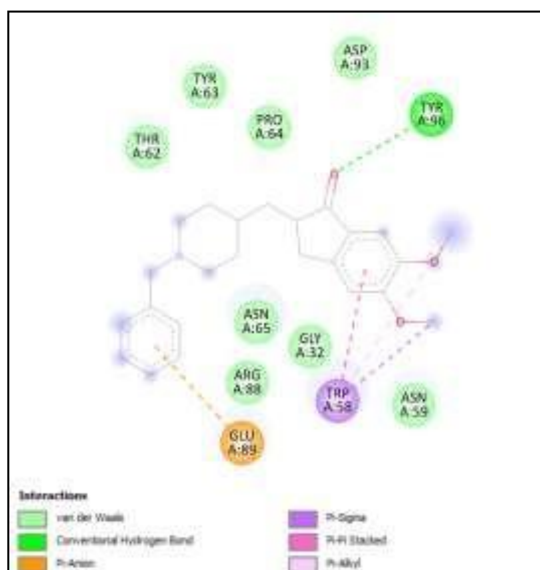
Table 1: Binding energy/Docking score of phytoconstituents with the target.

Sr. No.	Compound	Docking Score
1	Galantamine (Standard)	-6.1
2	Co-crystallized ligand	-6.5
3	Quercetin	-7.0

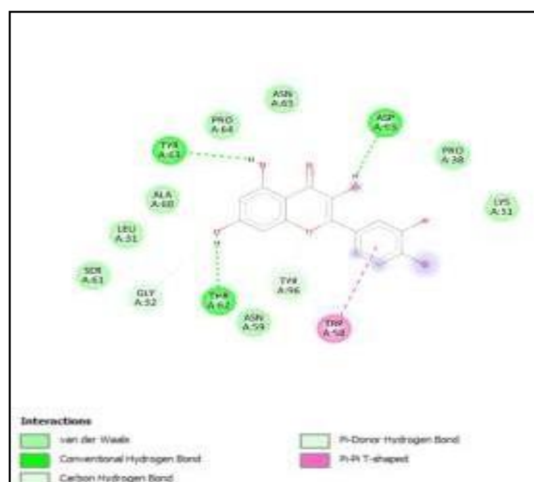
4	Crocetin	-5.3
5	4-Hydroxy Hexahydrobenzofuran-7-One	-4.8
6	Arborside-C	-4.8
7	Nyctanthoside.	-5.6
8	Kaemferol	-6.9

Table 2. Drug likeness properties calculation using Swiss ADME database.

Code	**MW**	**HB^d**	**HB^a**	**QPlogPo/w**	**PSA**	**Rule of five**
Quercetin	302	5	7	1.98	122.10	0
Crocin	977	14	24	-5.22	389.93	5
Crocetin	328	2	2	4.60	142.75	0
4-Hydroxy Hexa-hydrobenzofuran-7-One	156.181	1	3	0.11	65.35	0
Arborside-C	510.492	5	12	-1.31	206.15	3
Nyctanthoside.	422.383	7	12	-4.20	165.04	2
Kaemferol	286.239	4	6	2.28	117.31	1



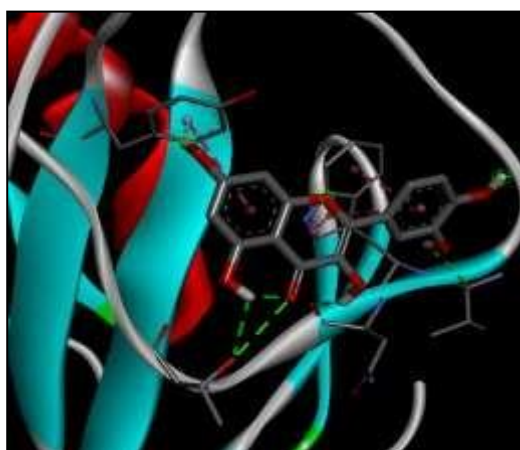
(A)



(B)

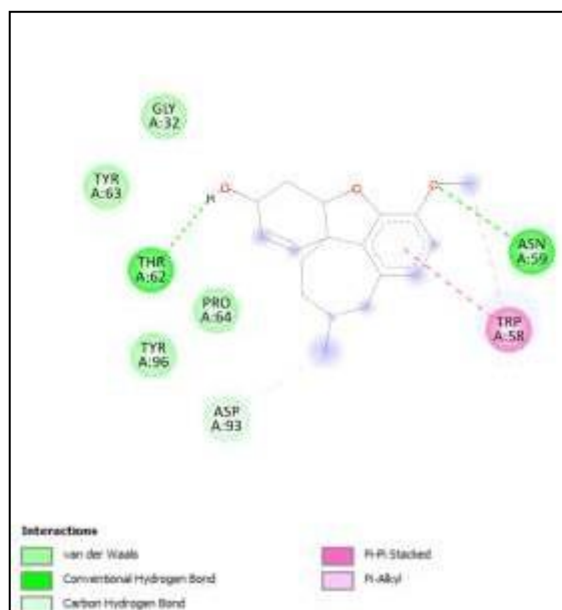
Figure 3. Ligand interaction diagram (2D). (A) The co-crystallized ligand (PDB ID: 1EVE) (B) Compound Quercetin with protein receptor (Best docked).

(C)

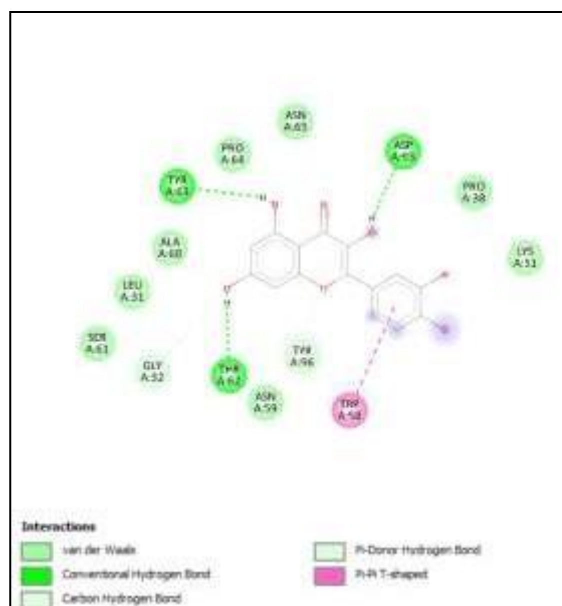


(D)

Figure 4. Ligand interaction in 3D for the co-crystallized (C) ligand (PDB ID: 1EVE) and (D) compound Quercetin with protein receptor.

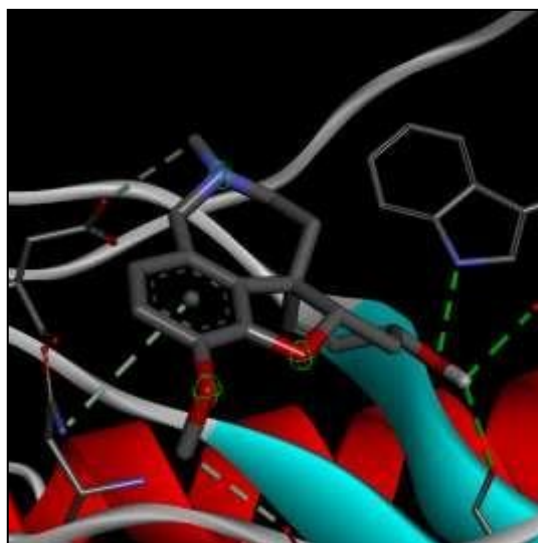


(A)

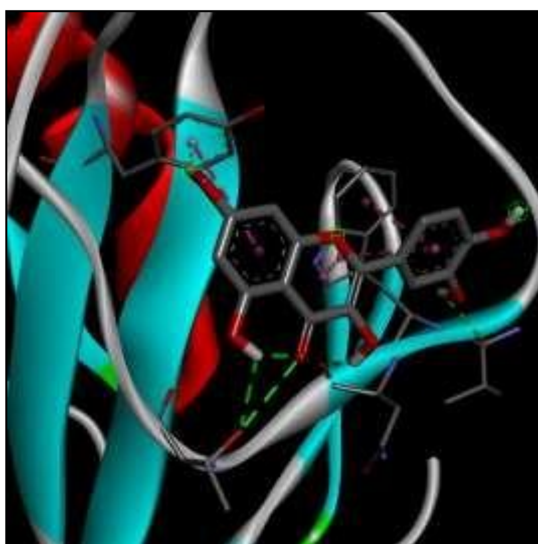


(B)

Figure 5. Ligand interaction diagram (2D). (A) The Galantamine (standard drug) (B) Compound Quercetin with protein receptor (Best docked).



(C)



(D)

Figure 6. Ligand interaction in 3D for the Galantamine (standard drug) (C) and compound Quercetin (D) with protein receptor (Best docked).

4. DISCUSSION

Oxidative stress plays an important role in inducing and activating multiple cell signaling pathways that results into formation of toxic substances and then promotes the development of Alzheimer's disease.[15] Reduction in cognitive functions in dementia is predominantly related to a decrease in cholinergic neurotransmission. Acetylcholine reduction is primarily responsible for initiation of AD. Cholinesterase (ChE) inhibitors enhance central cholinergic function by inhibiting the enzymes that degrade ACh, thereby increasing the availability of ACh to stimulate nicotinic and muscarinic receptors within the brain. Hence inhibitors of AChE are the standard approach to the symptomatic treatment of AD.[16,17]

Hence antioxidant and anticholinesterase activity are key factors to evaluate the anti-Alzheimer's potential of drug candidates. The measurement of antioxidant activity in foods and plants is performed by more than one *in vitro* method in order to establish the antioxidant ability of samples. therefore, *in-vitro* antioxidant activity of the ethanolic extract of *Nyctanthes arbortristis* was analyzed using the DPPH method and lipid peroxidation method. Antioxidants react with reactive oxygen species by inhibiting the enzyme or by chelating trace elements. The DPPH free radical assay can be considered reliable and reproducible. The neutral DPPH radical was used to evaluate the radical scavenging activity. The study revealed that EENA was able to reduce the radical DPPH to the yellow-coloured diphenylpicrylhydrazine in a concentration-dependent manner. DPPH is a stable free radical at room temperature, which produces a violet solution in ethanol. DPPH shows an absorption maxima at 517 nm in the visible spectrum (deep violet colour). As the electron became paired in the presence of free radical scavenging the absorption decreases in a concentration dependant manner. The peroxiding of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. The antioxidant activity of EENA may be due to the presence of a hydroxyl group in phytoconstituents which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen. Lipid peroxidation is one of the major consequences of oxidative imbalance-mediated injury to the brain. It affects fluidity and permeability of cell membranes and impairs the activity of membrane-bound enzymes.[18] Researchers demonstrate that serum or plasma TBARS level in AD subjects is significantly higher than in controls.[19] Oxidative stress can lead to peroxidation of cellular lipids and can be measured by determining the levels of thiobarbituric acid reactive substances. MDA is a product of lipid peroxidation.[20] MDA appears to be the most mutagenic product of lipid peroxidation. The TBA test is predicated upon the reactivity of thiobarbituric acid toward MDA to yield an intensely coloured chromogen red complex which is measured at 532 nm. EENA significantly inhibit lipid peroxidation by a reduction in the red colour complex formed confirming its anti- lipid per oxidative potential.[21]
Acetylcholine is a key element of brain responsible for learning capabilities and dementia. Reduction in amount of acetylcholine is associated with Alzheimer's disease. AChE is found among neurofibrillary tangles and neuritic plaques. Acetylcholinesterase inhibitors are potent drug molecules that prevent the degradation of acetylcholine and hence acetylcholinesterase inhibitors are considered as an effective treatment for Alzheimer's disease.[22] Hence, the AChE inhibitory effects of EENA indicate their potential in the development of natural therapeutics for Alzheimer's disease and related problems.

Based on the results, it can be concluded that the EENA has strong antioxidant and anticholinesterase activity, evidenced by the free radical scavenging and reducing power property, which may be due to the presence of carotenoids and flavonoids components in the extract.

5. CONCLUSION

The plant extract is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity promoter. EENA showed potent antioxidant activity and Acetylcholinesterase inhibitory effect, which is useful in Alzheimer's targets. From the molecular docking results, it can be inferred that compound Quercetin acts in a similar way as that of Galantamine to show a potent anti-Alzheimer's activity. The ADMET properties calculated also satisfied Lipinski's rule of five violations to behave like a drug. The toxicity data clarify the safety of the drugs in a better way.

This study provides a baseline for the use of EENA for preclinical studies to evaluate efficacy in Alzheimer's disease.

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