Original Research Article

EVALUATION OF *CHENOPODIUM QUINOA* EXTRACT ON DIAZEPAM INDUCED MEMORY IMPAIRMENT IN ANIMAL MODELS

ABSTRACT

Aim: The purpose of this research is to look at the pharmacological and *insilico* studies of *Chenopodium quinoa*. Materials and Methods: The screening of *in vivo* anti- amnesic activity of the ethanolic seed extract of *Chenopodium quinoa* was carried out using Actophotometer and Rotarod apparatus. *In silico* approaches like docking studies (Mcule software) and Ramchandran plot (procheck), online softwares were used in the study to establish mechanism of action of active constituents present in the extract. Results: The extract treated groups at doses (200 mg/kg and 400 mg/kg, bd.wt) showed significant anti-amnesic activity. The basal activity score in actophotometer is as an indicator for impairment of learning and memory. Fall of time by rotarod is used to evaluate learning and memory in rodent models of CNS disorders as in case of amnesia. The results revealed that quercetin, kaempferol, myristic acid, palmitic acid, stigmasterol, lenolenic acid, pentadecanoic acid, tocopherols, arachidonic acid and standard donepezil have got highest glide scores against selected PDB ID: 1EVE, 2FY4, 7CUM and 3EJ8. Conclusion: From *in vivo* and *in silico* results it is evident that ethanolic seed extract of *Chenopodium quinoa* possessed significant anti-amnesic activity.

KEYWORDS: Donezepil, *Chenopodium quinoa*, Docking studies, Mcule software and Ramachandran plot

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1. INTRODUCTION

Alzheimer's disease is the most common cause of dementia – a continuous decline in thinking, behavioural and social skills that affects a person's ability to function independently due to abnormal build-up of proteins in and around brain cells. One of the proteins involved is called amyloid, deposits of which form plaques around brain cells where, the other protein tau deposits of which form tangles within brain cells. Progressive disease that destroys memory and other important mental functions [1]. Brain cell connections and the cells

themselves degenerate and die, eventually destroying memory and other important mental functions. The prevalence was higher in older age groups (75 years and above) as compared to those below 75 years of age. However, the prevalence rates were similar for males and females in rural and urban population. The current standard care drug therapy for AD is cholinesterase inhibitor donepezil [2].

Donepezil is widely used in clinical practice for Mild Cognitive Impairment –Alzheimer's Disease (MCI-AD) since in routine clinical practice; it is challenging to differentiate mild AD from MCI-AD without a detailed neuropsychological evaluation including assessment of activities of daily living. The ability of the Benzodiazepines is known as "acquisition-impairing" molecules, and their effects on anterograde memory processes are well described. Diazepam has well-known amnesic properties. These effects, however, are selective for certain psychobiologically distinct memory functions and a highly differentiated unfolding of cognitive impairment in response to increasing doses of diazepam. Diazepam produced a marked deficit in episodic memory, yet despite this, dense amnesia with selectively impaired anterograde episodic memory and attention while totally sparing access to information in long-term memory (semantic or knowledge memory) [3]. *Chenopodium quinoa* is an endemic plant peculiar to South America belonging to the family (*Chenopodiaceae*). Seeds of *quinoa* are used as antimicrobial, antioxidant, anti-inflammatory, anti-tumor and anti-carcinogenic effects. The present study aimed to evaluate the neurobehavioral and neuroprotective effect of the ethanolic extract of *Chenopodium quinoa* on Diazepam– induced amnesia in mice.

Comment [U1]: is necessary to include if there are some studies related to quinoa extracts and its application in pharmacological and *in silico* studies.

2. MATERIALS AND METHODS

2.1 Plant collection and drying

Seeds of *Chenopodium quinoa* were collected from the local market during the month of December 2020. This Material was identified and authenticated by Botanist Dr. P. Suresh Babu, lecturer, New Government Degree College, kukatpally. The marketed seeds were shade dried for a week and coarsely powdered in a mixer grinder. The powdered material was subjected for extraction process.

Comment [U2]: What variety of quinoa was used in the study?

Comment [U3]: It is necessary to describe the process of grain drying (the temperature, time, etc).

2.2 Preparation of ethanolic extract of *Chenopodium quinoa* (*Soxhlet)*

The powdered material of seeds of *Chenopodium quinoa* were dried and extracted with ethanol by soxhlation technique. As to get efficient extraction, this method allows a continuous extraction process; it is nothing but a series of short macerations. The organic extract obtained were evaporated to dryness by keeping at room temperature. Large amounts

Comment [U4]: What amount of ethanol was used in the extraction? How many macerations were made in the extraction? the ethanol was pure or in solution with water? Which were the conditions of ethanol evaporation?)

Comment [U5]: What is the room temperature?

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of drug can be extracted with a much smaller quantity of solvent. This process of extraction is economical in terms of time, energy and consequently financial investments.

2.3 Preliminary phytochemical analysis of the extract

The extract was subjected to preliminary phytochemical investigations to identify various phytoconstituents present in the ethanolic seed extract of *Chenopodium quinoa*.

Comment [U6]: What compounds were determinate with phytochemical analysis?
What methodology was applied for preliminary characterization?

2.4 Identification of Phytochemical constituents using Gas Chromatography:

The extract was subjected to GC-MS studies to identify the exact phytochemical constituents. GC-MS analysis were carried out by Agilent 6890 series GC-MS instrument coupled with mass spectroscopy as detector. Temperature was adjusted to -30°C – 280/300°C. The HP -5MS Column with dimensions 30 m×0.32 mm× 0.25µ m were used for analysis. This Oven temperature were adjusted to 35 °C and hold time 5 min, ramp 10°C/min up to 220°C Column flow is 1.2 ml. The inlet temperatures were kept at 250°C and the source temperature of 230°C and MS Quard temperature of 150 °C [4].

2.4.1 GC –MS conditions during analysis

2.4.1.1 GC condition

Column oven : 35 °C initial, hold
Temperature : Time 5 min
Injector Temperature: 250°C
Column Flow : 1.2 ml/min
Carrier Gas : Helium 99.9995% purity
Injection Volume : 1 microliter

2.4.1.2 MS condition

Ion source temp : 230 °C
MS quard : 150°C
Ionization EI : (-70 ev)
Scan speed : 2000

2.5 Acute toxicity testing

The acute toxicity studies were carried out using OECD 425 guidelines. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, and Hyderabad, India. (Reg.No. 1175/PO/ERe/S/08/CPCSEA) [5].

2.6 Animal housing

The animals (mice) were housed in poly acrylic cages with not more than six animals per cage, with 12 h light/12 h dark cycle. Animals have free access to standard diet and drinking water *ad libitum*. The animals were allowed to acclimatize the laboratory environment for a week before the start of the experiment. The care and maintenance of the animals were carried out as per the approved guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA).

***2.7 In vivo* methods for evaluation of anti-amnesic activity**

In vivo evaluation of anti-amnesic activity of the ethanolic extract of seeds of *Chenopodium quinoa* was carried out in following models.

1. Basal activity by Actophotometer
2. Fall of time by rotaroad test

2.7.1 Basal activity by Actophotometer

30 healthy albino mice of either sex weighing 25-30 gm were selected for the study. They were divided into five groups of 6 animals each. The actophotometer consist of a square arena (30 × 30 × 25 cm) with wire mesh bottom, in which the animal moves for 5 minutes. Six lights and six photocells were placed in the outer periphery of the button in such a way that mice can block only one beam. The movement of animal interrupts a beam of light falling on a photocell during which a count was recorded and displayed.

Group I: received (control) normal saline.
Group II: received (disease control) Diazepam (1.0 mg/kg, *i.p*).
Group III: received EECQ at dose of 200 mg/kg, *p.o.*
Group IV: received EECQ at dose of 400 mg/kg, *p.o*.
Group V: received Donepezil 1mg/kg, *i.p*

Respective drugs were administered to all groups 1hour before the trials. The basal activity score of each animal will be noted on 8^{th} and 9^{th} day. The difference in the activity will be recorded considering standard drug treatment score and extract treatment score [6].

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Figure 1: Basal activity by Actophotometer

2.7.2 Fall of Time by Rotarod Test

30 healthy albino mice of either sex weighing 25-30 gm were selected for the study. They were divided into five groups of 6 animals each. For the training trials, the mice will be placed on the rotarod at 25 rpm for about 10 minutes per day. Respective drugs were administered to all groups 1hour before the trials.

Group I received (control) normal saline.

Group II: received (disease control) Diazepam (1.0 mg/kg, *i.p*).

Group III: received EECQ at dose of 200 mg/kg, *p.o.*

Group IV: received EECQ at dose of 400 mg/kg, *p.o*.

Group V: received Donepezil 1mg/kg, *i.p*.

Mice will be initially selected for the ability to remain on the rotating bar rotating at a constant speed of 25 rpm for at least two consecutive 180 seconds trial. Measure the fall time of individual animals using the same conditions and noted on 8^{th} and 9^{th} day of the trial [6].

Figure 2: Fall of Time by Rotarod Test

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2.7.3 Histopathological Studies

The mice brain was collected and isolated with formalin solution 10 %. Then, the brains were routinely embedded in paraffin and stained with haematoxylin eosin. The hippocampal lesions were assessed microscopically at 40 magnification [7].

2.8 Statistical Analysis

The results are reported as the mean ± SEM (n=6) of the mean analysis of variance followed by the Dunnetts multiple comparison tests which were used for comparison. Differences were considered significantly at $p< 0.05$.

2.9 *In silico* analysis

2.9.1 Molecular Docking Studies

2.9.1.1 Structure based drug design

Initially the protein downloaded from PDB was prepared by removing chain B. Water molecules present in both the chains are removed. Energy minimization was done. Later molecules drawn using chemdraw were converted to mol format and ligprep was created. Grid generation was done by removing crystal ligand and the structures were docked against protein 1EVE, 2FY4, 7CUM and 3EJ8.

2.9.1.2 Mcule docking results

Mcule docking software was used in the present study. The selected proteins are Acetyl choline esterase inhibitor (PDB ID: 1EVE), choline acetyltransferase inhibitor (PDB ID: 2FY4), GABA inactivator (PDB ID: 7CUM) and NOS inhibitor (PDB ID: 3EJ8).

2.9.2 Ramachandran plot

Ramachandran plot has been generated from PROCHECK validation server which was used to access the quality of the model by looking into the allowed and disallowed regions of the plot [4]

3. RESULTS AND DISCUSSION

Ethanolic extract of *Chenopodium quinoa* was explored for its *in vivo* anti-amnesic activity using suitable rodent models and *in silico* analysis using Mcule software. All the results obtained in the study were included below.

3.1 Preparation of ethanolic extract of seeds of *Chenopodium quinoa*

The ethanolic extract of seeds of *Chenopodium quinoa* was prepared by soxhlation technique. The percentage yield of ethanolic extract was calculated by using the following formula.

% of yield obtained=Amount of extract obtained (gm)/ Total amount powder used (gm) ×

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100

% Yield of extract=46.990/140 ×100=33.5%

3.2 Preliminary phytochemical analysis

The preliminary phytochemical investigation of ethanolic extract of seeds of *Chenopodium quinoa* revealed the presence of bioactive compounds of which flavonoids, glycosides, terpenoids, phenolic compounds, sterols, tannins and proteins were the most prominent (Table 1).

Comment [U7]: Describe the identification of compounds found in the chromatogram.
What kind of compounds were found in the chromatogram?

Table 1: Preliminary phytochemical analysis.

Phytoconstituents	Results
Flavonoids	+
Saponins	+
Terpenoids	+
Phenolic compounds	+
Tannins	+
Proteins	+
Sterols	+

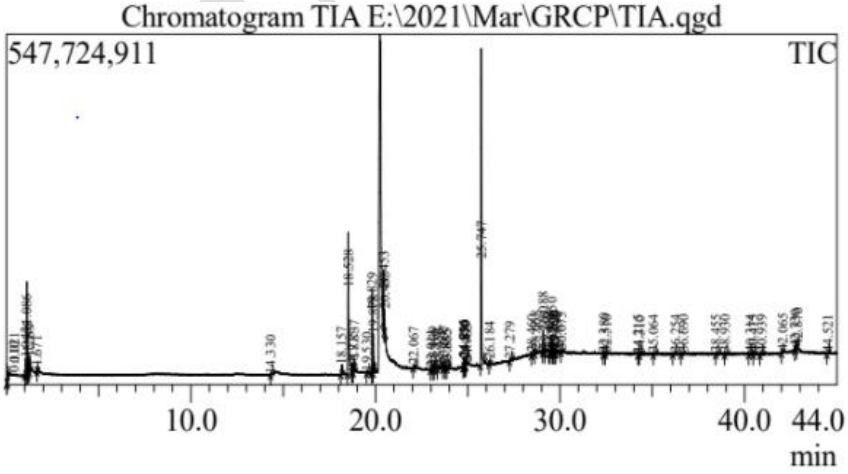

Figure 3: Identification of phytoconstituents using gas chromatography

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3.3 Acute toxicity studies

Ethanolic extract of seeds of *Chenopodium quinoa* was tested on Swiss albino mice up to a dose of 2000 mg/kg bd. wt. The animal did not exhibit any signs of toxicity or mortality up to 2000 mg/kg bd. wt. Various morphological and behavioral characters were observed during the study. Hence the extract was found to be safe up to 2000 mg/kg bd. wt.

3.4 Dose selection

From toxicity studies, a dose of 2000 mg/kg bd. wt. was identified to be safe, and the working dose was considered as $1/10^{th}$ i.e., 200 mg/kg. bd. wt. In the present study pharmacological evaluations were done using 200 mg/kg. bd. wt. and 400 mg/kg. bd. wt. of the extract.

3.5 *In vivo* anti-amnesic activity

The ethanolic extract of seeds of *Chenopodium quinoa* was screened for its anti-amnesic activity using the following models.

3.5.1 Basal Activity Score using Actophotometer Apparatus

Table 2: Effect of EECQ on Basal Activity Score by Actophotometer Apparatus

Groups	8^{th} Day	9^{th} Day
Control	473±3.0	474.5±3.0
Disease control diazepam (1 mg/kg bd. wt)	179±3.0**	180±2.0**
EECQ (200 mg/kg bd.wt)	310±4.0^{**aA}	311±4.0^{**aA}
EECQ (400 mg/kg bd.wt)	361±5.0^{**aA}	383±3.0^{**aA}
Donepezil (1.0 mg/kg bd.wt)	447±3.0^{*a}	448±3.0^{**a}

Values are expressed as mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test by comparing with control, disease control & standard. Significant values are expressed as control group (** = p< 0.0001, *= p<0.0005), disease control group (a=p<0.0001) & Standard group (A = p<0.0001)

3.5.2 Fall off time by Rotarod Apparatus

Table 3 Effect of EECQ on fall off time by Rotarod Apparatus

Groups	Fall off time (secs)	
	8^{th} day	9^{th} day
Control	187.3±1.2	188±1.2

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Disease control	72.5±1.6*	78±1.0*
Clonazepam (1 mg/kg bd. wt)		
EECQ (200 mg/kg bd.wt)	100±1.4*aA	103±1.5*aA
EECQ (400 mg/kg bd.wt)	175±1.1*aA	176±0.8*aA
Donepezil (1.0 mg/kg bd. wt)	162±0.7*a	166±0.7*a

Values are expressed as mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test by comparing with control, disease control & standard. Significant values are expressed as control group (* = p< 0.0001), Disease control group (a = p<0.0001) & Standard group (A = p<0.0001).

The human amnestic syndrome associated with lesions of the hippocampus and amygdala is characterized by a selective impairment of recent (explicit, episodic) memory. Benzodiazepine (BZD) treated normal subjects demonstrate similar, marked impairments in episodic memory, but in addition, BZD also induces sedation and inattention. Diazepam is well established as inhibitory modulators of memory processing. This effect is especially prominent when applied before the acquisition phase of a memory task. Explicit memory learning seems to be affected through the $GABA_A$ receptors containing the α_1 and α_5 subunits, the role as subunits, mainly expressed in the hippocampus, in modulating distinct forms of memory gives certain memory deficit states. The phytochemical constituent identified in the ethanolic extract of *Chenopodium quinoa seeds* are saturated fatty acid, phenols, sterols, flavonoids, terpenoids, tannins, proteins and anti-oxidant [8].

α–linolenic acid increases hippocampal m-RNA levels and specific phospholipase A2 encoding–gene in brain and Aβ 1-42 Inhibited Aβ oligomerization decrease tau phosphorylation and pro-apoptotic proteins that leads in improvement in the cognitive function through the activation of extracellular signal-regulated kinases (ERK) and Akt signaling in the rat model increase the basal activity score by interrupting the beam of light in the arena [9].

P-Coumarin activates the GABA–A receptor as it is a phenolic compound and it shows high anti-oxidant property which help in decreasing the oxidative stress and holding the rod for longer time reducing the fall of time [10]. Kaempferol act as anti-oxidant and decreases the aggregation of beta amyloid and also reduces the age-related memory impairment and increases protective responses to oxidative stress and mitochondrial dysfunction. It is potential of protection of neurons against injuries induced by neurotoxins and promotion of learning and memory leading to extend the fall of time [11].

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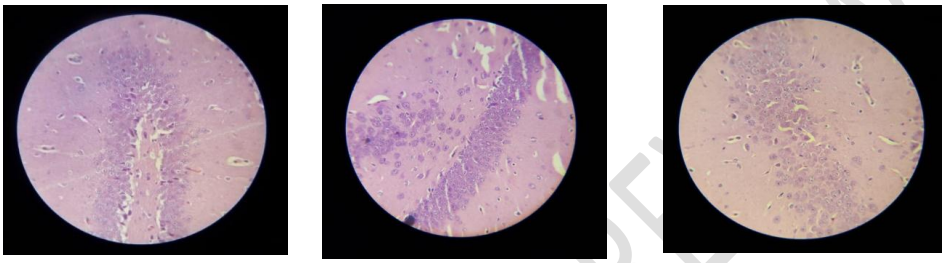
Vitamine-E (Tocopherol) prevent $A\beta_{1-42}$ induced protein oxidation, ROS production in hippocampus and frontal cortex leads to increase in the memory and neurotoxicity in primary rat embryonic hippocampal neuronal culture. Memory deficits and apoptosis which are likely to be seen in amnesia were prevented by vitamin E [12].

Donepezil binds reversibly to acetylcholinesterase and inhibits the hydrolysis of acetylcholine, thus increasing the availability of acetylcholine at the synapses, enhancing cholinergic transmission [13].

3.6 Histopathology Studies



GROUP I: Normal control **GROUP II:** Disease control **GROUP III:** EECQ (200 mg/kg, bd. wt, *p.o*)

GROUP IV: EECQ (400 mg/kg, bd. wt, *p.o*) **GROUP V:** Donepezil (1 mg/kg, bd. wt, *p.o)*

Figure 4: Histopathology studies showing arrangement of different layers of pyramidal cells in all groups except in group II where apoptic cells are observed in hippocampus region.

Group I: Control group – A compactly arranged 7-8 layer of pyramidal cells with prominent nucleus was observed in Hippocampus

Group II: Disease control group – Absence of pyramidal cells and presence of apoptotic cells was observed in Hippocampus

Group III: EECQ (200 mg/kg) - Irregular arrangement of 2-3 layers of pyramidal cells with scattered pattern and mild appearance of apoptotic cells was observed in Hippocampus

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Group IV: EECQ (400 mg/kg) – Neuronal cells are well organized with 4-5 layers of pyramidal cells was observed in Hippocampus

Group V: Donepezil (1 mg/kg) - Neuronal cells are well organized with 6-7 layers of pyramidal cells and absence of apoptotic cells was observed in Hippocampus

3.7 *Insilico* analysis

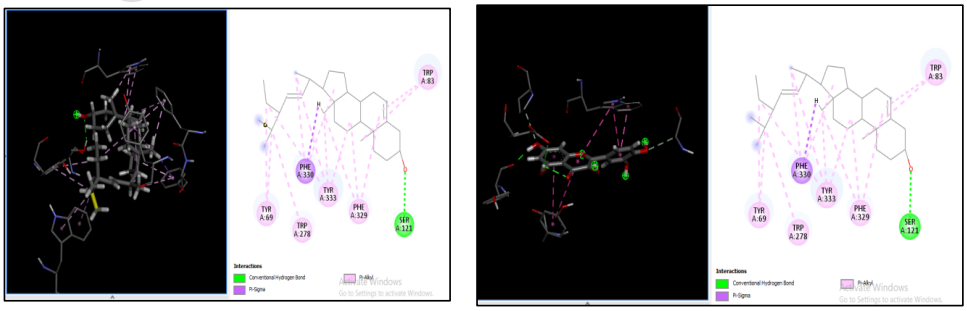
i) Molecular docking

Table 4: Schrodinger XP Docking Score

Sl.no	Compounds	1EVE	2FY4	7CUM	3EJ8
1	Tetradecanoic acid/ Myristic acid	-6.5	-4.6	-5.2	-5.0
2	Hexadecanoic acid /Palmitic acid	-6.9	-4.9	-5.5	-5.8
3	Eicosadienoic acid	-7.5	-5.1	-6.3	-7.0
4	Pentadecanoic acid	-7.3	-4.7	-5.2	-6.2
5	Tocopherols	-9.9	-6.5	-7.5	-8.1
6	Stigmasterol	-11.9	-6.4	-8.4	-9.6
7	Beta sitosterol	-9.1	--	-6.2	-5.6
8	Quercetin	-10.4	-7.3	-9.3	-7.8
9	Benzoic acid	-6.3	-4.7	-4.5	-5.2
10	Kaempferol	-10.2	-4.6	-8.5	-7.5
11	Arachidonic acid	-7.7	-5.3	-6.8	-6.0
12	2-Bromotetrade canoic acid/2-Bromodecanoic acid	-6.5	-4.6	-5.2	-8.1
13	Donepezil	-10.9	-7.6	-8.8	-8.7

G score = glide score, The more negative the Glide score, the more favorable the binding

PDB ID: 1EVE



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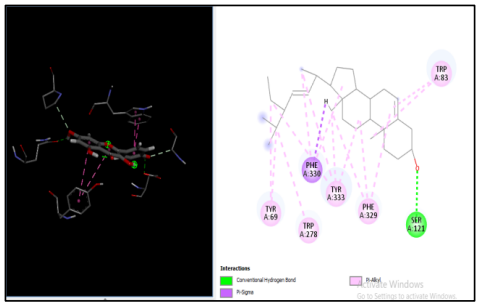
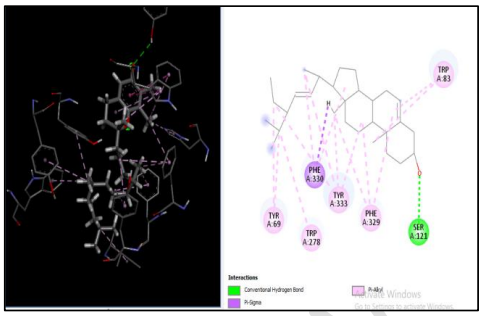
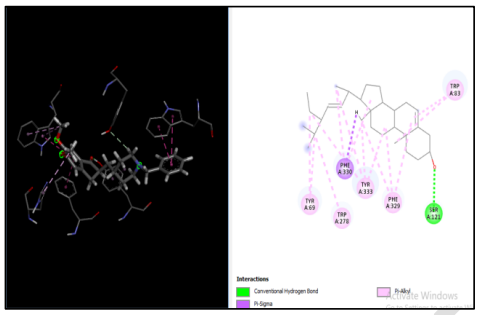
a) Stigmasterol – 11.9



b) Quercetin – 10.4



c) Kaempferol- 10.2



d) Tocopherol – 9.9

e) Donepezil-10.9

PDB ID: 2FY4

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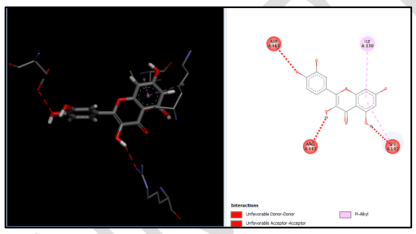
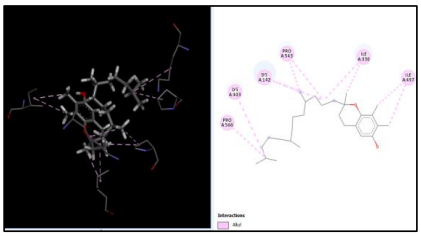
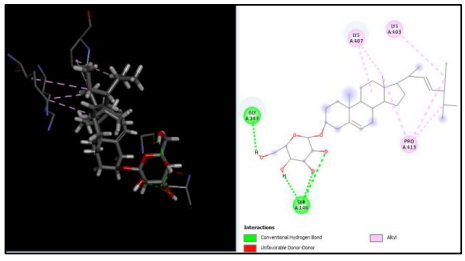
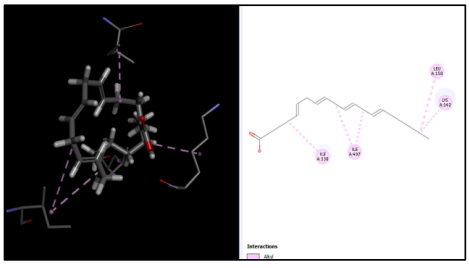


a) Quercetin -7.3



b) Tocopherol -6.5





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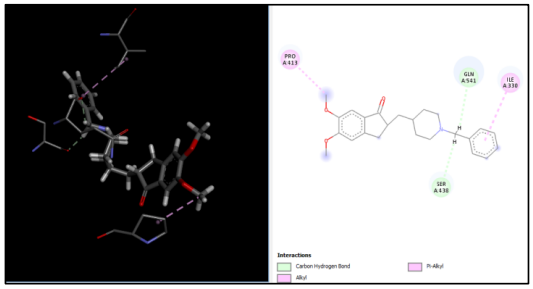
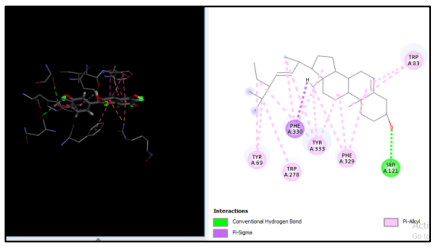
c) Stigmasterol -6.4

d) Arachidonic acid -5.3

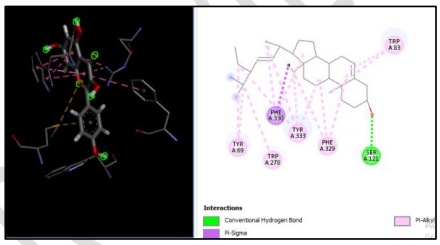


e) Donepezil -7.6

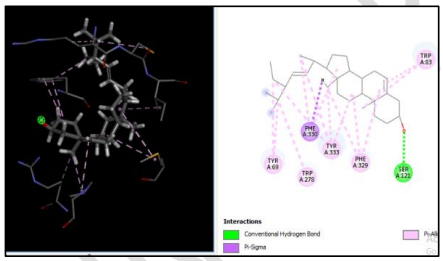
PDB ID:7CUM



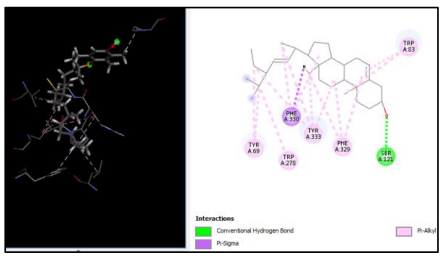
a) Quercetin – 9.3



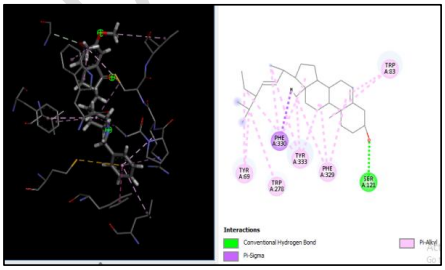
b) Kaempferol- 8.5



c) Stigmasterol – 8.4

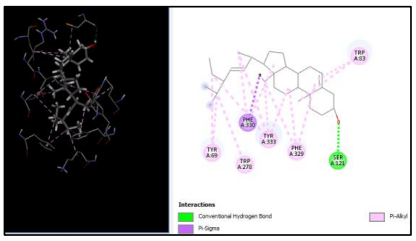


d) Tocopherol – 7.5

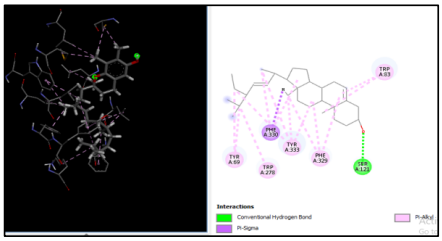


e) Donepezil-8.8

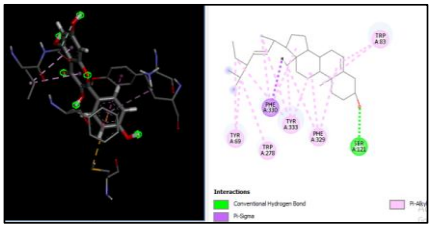
PDB ID:3EJ8



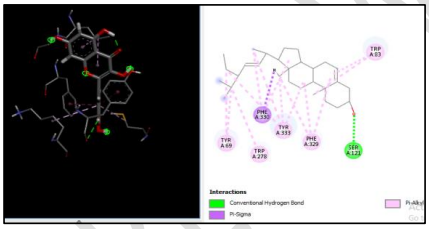
a) Stigmasterol – 9.6



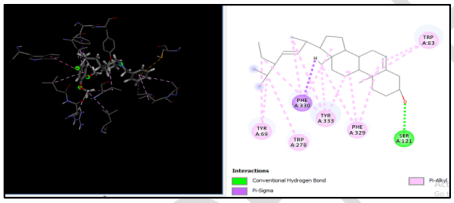
b) Tocopherol – 8.1



c) Kaempferol- 7.5



d) Quercetin – 7.8



e) Donepezil- 8.7

ii) Ramachandran plot Analysis

Protein 1EVE, 2FY4, 7CUM and 3EJ8 were analysed for Ramachandran plot to know amino acid presence in different regions of respective protein tabulated in table 5 and pictorial representation which is given in figure 5 below.

Table 5: Ramachandran plot status with protein with 1EVE, 2FY4, 7CUM and 3EJ8

Residues	1EVE	2FY4	7CUM	3EJ8
Most favourable region (%)	87.3	91	83.3	87.4
Additional allowed regions (%)	11.8	8.8	15.9	12.2
Generously allowed regions (%)	0.7	0.0	0.3	0.4

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Disallowed regions (%)	0.2	0.2	0.0	0.0

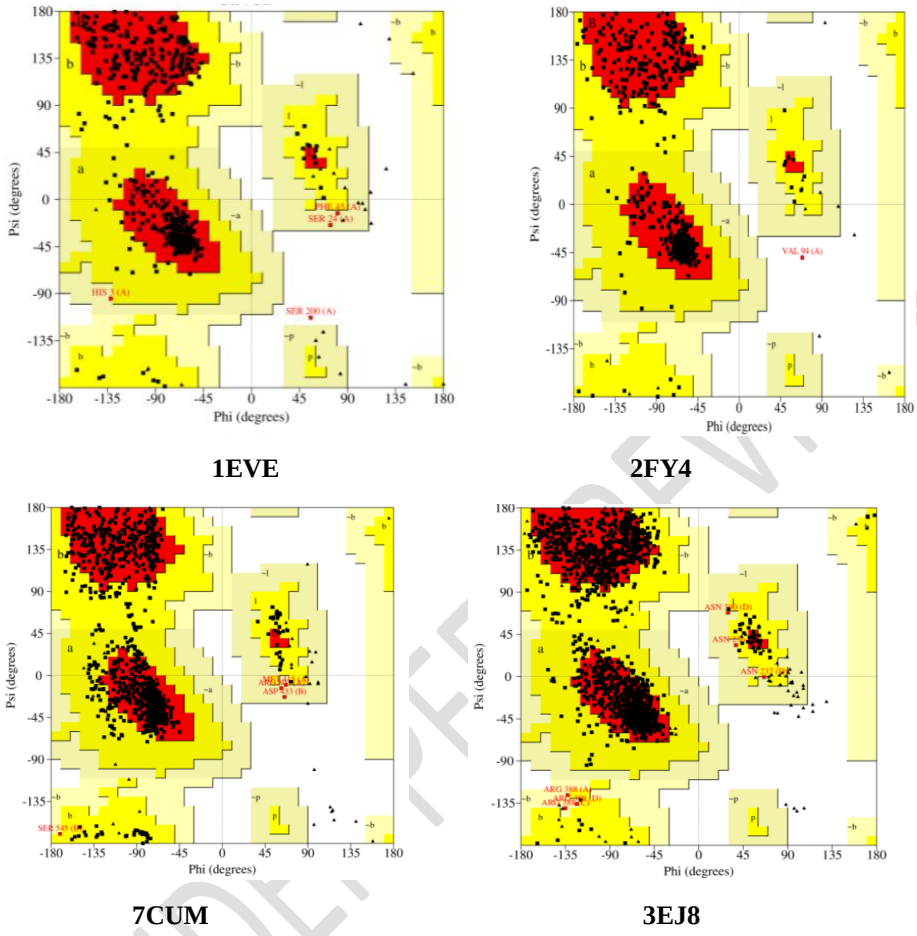

Figure 5: Ramachandran plot of protein 1EVE, 2FY4, 7CUM and 3EJ8

Molecular docking continues to holds great promise in the field of computer-based drug design which screens small molecules by orienting and scoring them in the binding site of a protein. The docking analysis of isolated compounds from ethanolic extract of seeds of *Chenopodium quinoa and* standard donepezil were carried out using Mcule software. The various constituents identified in the plant extract are myristic acid, palmitic acid, eicosadienoic acid, pentadecanoic acid, tocopherols, stigmasterol, β-sitosterol, quercetin, benzoic acid, kaempferol, arachidonic acid, benzofuran, 2-bromodecanoic acid and standard donepezil were subjected to docking against PDB ID: 1EVE, 2FY4, 7CUM and 3EJ8. The

highest glide scores were observed with quercetin, kaempferol, myristic acid, palmitic acid, stigmasterol, pentadecanoic acid, tocopherols, arachidonic acid and standard donepezil against PDB ID: 1EVE, 2FY4, 7CUM and 3EJ8. The glide scores of the quercetin, and kaempferol, were found to be more than the glide score of standard drug donepezil against all selected proteins stating that the compounds might have same affinity to bind to the proteins. These results clearly indicate that the chemical constituents mentioned above might have shown similar mechanism to that of the standard drug donepezil as an anti-amnesic. The proteins identified namely PDB ID: 1EVE, 2FY4, 7CUM and 3EJ8 are modelled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is evident from the Ramachandran plot that predicted models have most favorable regions, additionally allowed regions, generally allowed regions and disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted models are of good quality. According to Ramachandran plot a good quality model would be expected to have over 90% in the most favoured region. Proteins like PDB ID: 1EVE, 2FY4, 7CUM and 3EJ8 showed almost 80-90% favoured region which clearly indicates that the selected models in the present study are of good quality [14].

4. CONCLUSION

Comment [U8]: Improve the conclusion.

From *in vivo* and *in silico* analysis the ethanolic extract of seeds of *Chenopodium quinoa* possessed anti-amnesic activity in rodent models. Further studies are needed to be carried out to isolate individual phytochemical constituents of the extract and to establish the exact mechanism for its anti-amnesic activity.

CONSENT

Not applicable

ETHICAL APPROVAL

The ethical approval for the study was taken as per the IAEC and the approval number is GRCP/COL/170219887010/2020.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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