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PHARMACOLOGICAL EVAUTIONS OF METHANOLIC EXTRACT AND FRATIONS OF *BUTEA MONOSPERMA*

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ABSTRACT

The i.c.v injection of Aβ peptide in negative control animals showed an extremely significant (P<0.001) increase in brain AChE levels. The AChE enzyme levels were significantly decreased in the treatment groups and indicated a difference with p<0.01, p<0.05, p<0.001 and p<0.05, respectively when compared with the amnesia induced group. The levels of each animal expressed in µmole/min/g. From the results, it was found that acetyl cholinesterase levels are increased in negative (0.23±0.015) when compared to control (0.10±0.0012), and decreased levels are observed in methanol extract (0.15±0.0026 and 0.13 ±0.0031 for 200 and 400 mg/kg, respectively) (Figure 4.8). The AChE enzyme levels were significantly decreased in the treatment groups of BME (0.15 ± 0.0026 and 0.12 ± 0.0023), BMC (0.19 ± 0.0025 and 0.16± 0.007), BMH (0.17 ± 0.0021 and 0.13 ± 0.0018) and BMQ (0.21 ± 0.0046 and 0.18± 0.0051) and indicated a difference with p<0.01, p<0.05, p<0.001 and p<0.05. Glutathione levels are decreased in negative (0.0159±0.00023) when compared to control (0.0298±0.00014), and levels are increased in methanolic extract of 200 mg/kg and 400 mg/kg respictively as follows 0.0266±0.00186 and 0.0275± 0.00 14. Glutathione levels are decreased in negative (0.0159±0.00023) when compared to control (0.0298±0.00014), and levels are increased in BME (0.0256±0.0018 and 0.0279 ± 0.0025), BMC (0.0239±0.0013 and 0.0253±0.0027), BMH (0.0247 ± 0.0008 and 0.0268 ± 0.0004) and BMQ (0.0227±0.0011 and 0.0242±0.0024) fractions.

KEYWORD: Respectively, Glutathione, animals, amnesia.

1. INTRODUCTION

Neuroprotection is a term used to allude to methodologies and relative components that shield the focal sensory system from neuronal wounds caused by incessant (e.g., Alzheimer's and Parkinson's ailments) or intense (e.g. stroke) neurodegenerative diseases (NDs) (Iriti *et al*., 2010). These intense or unending ailments result from break down and

crumbling of neurons of the central nervous system (CNS) and regularly result in the decay of the psychological and additionally the scholarly resources of the sufferers.

The beginning of NDs side effects is normally slow and in addition dynamic and incorporates loss of memory, fundamentally short term, trouble in learning, engine coordination, and numerous other practical loses (Adewusi *et al*., 2010; Nieoullon, 2011). Maturing, characterized as a complex physiological process including both morphological and biochemical changes that dynamically unfurl as we get more established (Iriti *et al*., 2010), has been observed to be intently connected with NDs. Maturing emerges as a noteworthy hazard factor among the other etiological elements of NDs, including hypertension, hereditary as well as ecological components and diseases. With expanding age, accumulation of proteins, irritation, oxidative stress, and loss of neurotransmitters, which are regular to the pathology of NDs, likewise happen all the more regularly (Iriti *et al*., 2010; Reeve *et al*., 2014; Hindle, 2010). Alzheimer's malady is the most widely recognized type of dementia in the maturity. The level of people with Alzheimer ailment increments by a factor of 2 with at regular intervals of age (Upadhyaya *et al.*, 2010). It influences about 5% of individuals 65-year old and more than 30% of those 85-year old. It is presently evaluated that there are 18−24 million individuals experiencing AD worldwide and this number is relied upon to reach 34 million by 2025 (Pan *et al.*, 2008). As indicated by the World Health Organization (WHO), 5% of men and 6% of lady of over the age of 60 years are influenced with Alzheimer's sort dementia around the world (Shrikant and kalpana, 2008) (Figure 1.1).

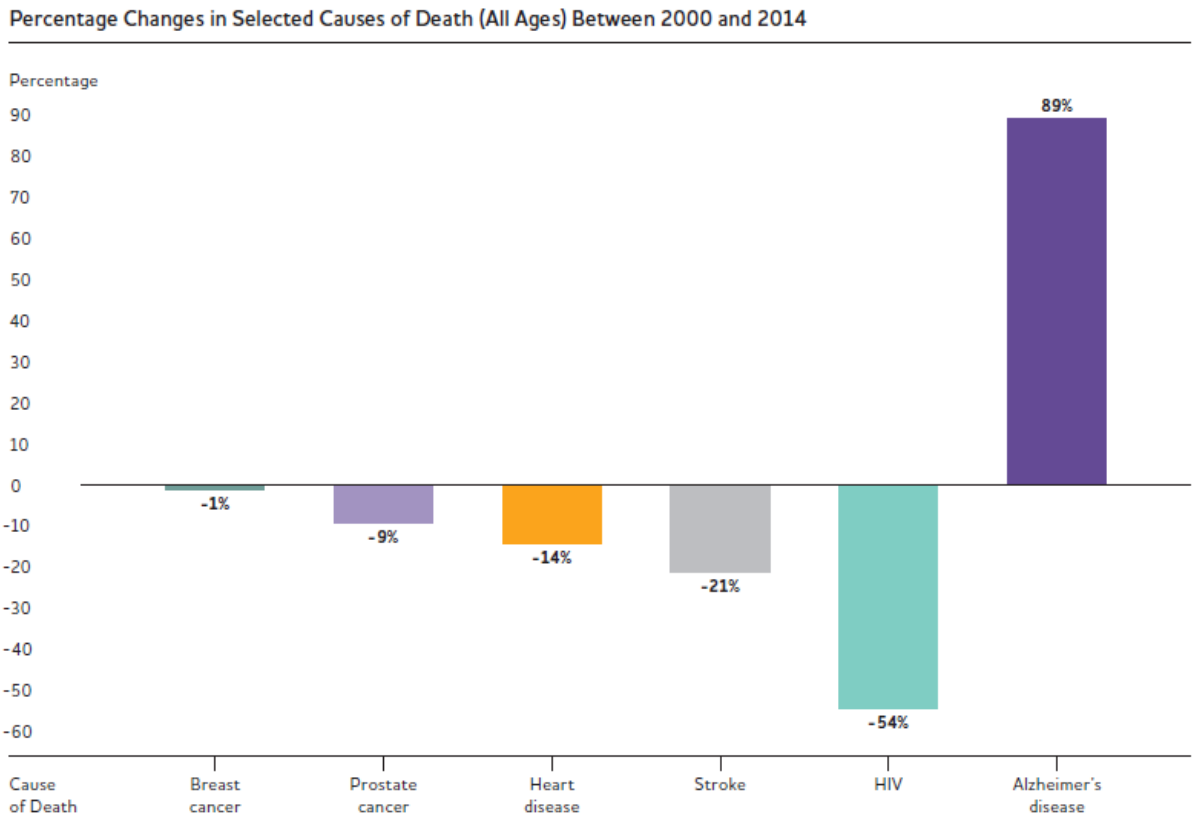

Figure 1.1: Epidemiology of AD.

2. PATHOPHYSIOLOGY

The Alzheimer's infection is described by three cardinal changes in the cerebrum (Figure 1.2).

(i) The development of dystrophic neurites around a focal center of amyloid (plaques),

(ii) The development of anomalous fibers (neurofibrillary tangles) made up of an exceedingly phosphorylated type of the microtubule-related protein tau in the perikaryia of specific neurons, joined by neuropil strings in axons and nerve terminals, and

(iii) Loss of defenseless neurons, primarily pyramidal, cholinergic, noradrenergic and serotonergic neurons (Palmer, 2002).

There are such a significant number of pathophysiological factors in charge of AD

i. Amyloid theory
ii. Tau protein theory
iii. The Cholinergic theory
iv. The part of free radicals and oxidative anxiety
v. The Glutamatergic speculation
vi. The part of aggravation
vii. Other chance variables (Blennow *et al.*, 2006).

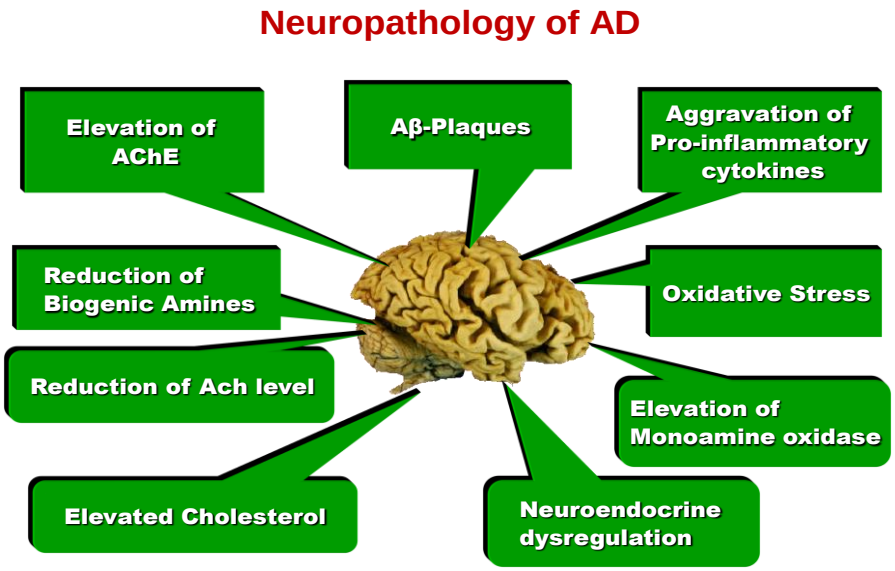

Figure 1.2: Neuropathology of AD.

1) AMYLOID CASCADE HYPOTHESIS

Beta-amyloid plaques are bunches of insoluble peptides that outcome from the anomalous cleavage of APP, the correct capacity of which is obscure. Application is ordinarily separated by 3 chemicals β secretase, γ-secretase, and α-secretase. Cleavage by β-secretase, trailed by γ-secretase, yields a dissolvable 40 amino corrosive peptide. In AD, a variation type of the γ-secretase divides APP at an erroneous place, making a 42 amino corrosive peptide called Aβ42 or Aβ, which isn't solvent and totals into identifiable bunches named β-amyloid plaques. Alpha-secretase really serves a defensive capacity as it separates APP at a site that averts Aβ arrangement (Figure 1.3).

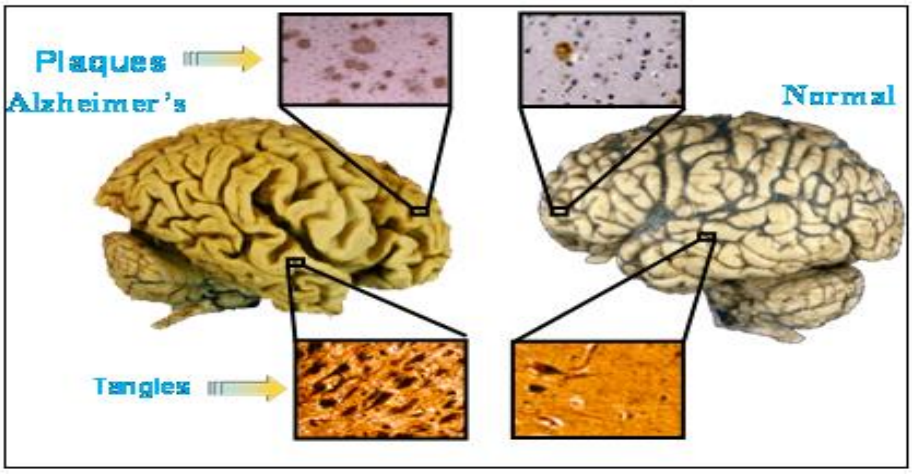

Figure 1.3: Amyloid plaques and neurofibrillary tangles in Alzheimer's disease and normal aging.

Two noteworthy leaps forward in regards to the etiology of AD accompanied the revelations that (i) amyloid stores of Aβ, a peptide sub piece of APP, are available in both diffuse and neuritic plaques, and (ii) transformations in APP, situated on chromosome 21, are in charge of many instances of familial AD. These discoveries plainly recommend that Aβ assumes a part in the etiology of AD, a hypothesis bolstered by information demonstrating that individuals with Down's disorder (trisomy 21) constantly create neurotic changes indistinguishable to those found in AD (Cork *et al.*, 2006).

Notwithstanding, the most well-known reason for familial AD is a transformation in PSEN1 (that encodes a part of a multisubunit proteolytic protein named γ-secretase); changes in PSEN2 (additionally a γ-secretase segment) are less normal. All changes known to cause familial AD are related with strange APP proteolytic preparing and prompt expanded creation of Aβ42, the most rich APP determined part of amyloid plaques. In any case, autosomal prevailing familial AD represents under 5% of all cases, and the factor or factors that trigger obsessive changes in the larger part of instances of AD (that are not hereditarily acquired) remain a secret (Hardy, 2009; Pimplikar, 2009).

2) TAU PROTEIN HYPOTHESIS

Neurofibrillary tangles are generally found in the cells of the hippocampus and cerebral cortex in people with AD and are made out of unusually hyperphosphorylated tau protein. The phosphoprotein, tau that is orchestrated by neurons is likewise present in astrocytes and oligodendrocytes inside the mind, and in fringe tissues. Tau is coded by a quality situated on chromosome 17 and on the other hand grafted into 6 isoforms extending from 352– 441 amino acids long. Tau protein gives basic help to microtubules, the cell's transportation and skeletal emotionally supportive network. Phosphorylation of tau protein is directed by the harmony between different kinases (e.g. GSK-3 and CDK5) and phosphatases (e.g. PP-1 and PP-2A) (Blennow *et al.*, 2006).

At the point when tau fibers experience strange phosphorylation at a particular site, they can't tie adequately to microtubules, and the microtubules crumple. Without an in place arrangement of microtubules, the cell can't work appropriately and inevitably passes on (Reynolds and Mintzer, 2005). Amid AD pathogenesis, tau proteins progress toward becoming hyperphosphorylated, upsetting their securities to microtubules, accordingly falling microtubule structure and decimating the neuron's vehicle and correspondence framework (Selkoe, 2002).

3) CHOLINERGIC HYPOTHESIS

The pathophysiology of AD is intricate and includes a few diverse biochemical pathways. The key manifestations of AD are principally caused by cholinergic brokenness. It is realized that ACh is a vital neurotransmitter identified with memory and learning (Nattanan *et al.*, 2009). The specific degeneration of cholinergic neurons that begin in the basal forebrain and activities to the cortex and hippocampus brings about the loss of all known cholinergic markers, for example, choline acetyltransferase (AChT), ACh levels and acetylcholinesterase (AChE). Ach is related with perception and it is the deficiency of this neurotransmitter which adds to intellectual brokenness (Small and Mayeux, 2005).

Then again, Acetylcholine is known to advance non-amyloidogenic preparing and diminish tau phosphorylation by lessening the action of protein kinase which phosphorylates tau. In this way, interruption of cholinergic flagging may prompt a criticism circle that expands creation Amyloid beta through adjusted APP handling, expanding phosphorylation of tau protein, in this way adding to the movement of AD pathology (Lahiri *et al.*, 2003).

PLANTS SELECTED FOR THE STUDY

Butea monosperma

2.1.3. PLANT INTRODUCTION

2.1.3.1. DESCRIPTION OF *BUTEA MONOSPERMA*

Botanical Name: ***Butea monosperma***

Family: ***Fabaceae***

Vernacular names:

English – Bastard Teak

Hindi – Dhak

Sanskrit – Palasa

Telugu – Moduga Chettu

Phyto constituents: Glucoside, kano-oil, palmitic acid, lignoceric acid, triterpene, butein, isobutrin.

Traditional use: Leprosy, expectorant, diuretic, gonorrhea, warms, astringent, antihelmintic.

2.1. MATERIALS

Acetylthiocholine iodide, dithiobisnitro benzoic acid, reduced glutathione, Acetyl β-amyloid (25-35) peptide(Catalog number SCP0002) are purchased from Sigma Aldrich, USA. Other reagent solvents including ethyl acetate, methanol, chloroform and N-hexane used were analytical grade purchased from Himedia laboratories, Hyderabad, India. diphenyl picryl hydrazyl (DPPH), L-ascorbic acid, thiobarbituric acid, sodiumdodecyl sulphate, tetra ethoxy propane, pyrogallol, ammonium molybalate, disodium hydrogen phosphate, sodium azide, dinitro phenyl hydrazine, sodium azide, trichloro acetic acid, donepezil were purchased from Hi media Labs, Hyderabad, India. All other reagents used were also of analytical grade.

2.1.1. List of equipments used for the study

- Medicraft Jumping box (INCO, Model No: 506/1)
- Y-maze video tracking system (VJ Instruments)
- Medicraft Rectangular maze (INCO, Model No: 511 6R)
- Centrifuge (Biofuge)
- Stereotaxic apparatus (INCO)
- Homogeniser (Remi)
- Spectrophotometer (Systronics)
- Desiccator
- Soxhlet apparatus

2.1.2. EXPERIMENTAL ANIMALS

Swiss albino mice (male) weighing 22-25 g at the age of 5-6 weeks, obtained from Jeeva life sciences, Hyderabad was used for the pharmacological studies. The animals were housed in polypropylene cages under standard conditions maintained at 23-25^{o}C, 12h light /dark cycle and given standard pellet diet (Vyas Labs, Hyderabad) and water *ad libitum.* The animals were acclimatized to the laboratory conditions for a week prior to the experimentation. All the animals handling and experimentation were conducted in accordance with the prior approved guidelines from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and the experimental protocol was approved by Institutional Animal Ethical Committee (Sura/05/Hyd /2024).

3.1. STUDIES ON METHANOLIC LEAF EXTRACT AND FRACTIONS OF *BUTEA MONOSPERMA*

3.1.1. Plant material collection

The leaves of *BM* were collected from the Kakatiya University, Warangal, Telangana, India, during month of May and authenticated by botanist Prof. Mustafa, Department of Botany, Kakatiya University, Warangal, Telangana, India.

3.1.2. Extraction

The collected leaves of *BM* was shade dried, powdered and extracted with Methanolic solvent by using Soxhlet apparatus. Further, the methanolic extract of leaves of *BM* were fractionated with ethyl acetate, n-Hexane, chloroform and water as solvents. The extract of *MEBM* were dispersed in 1 L of distilled water separately and fractionated with ethyl acetate, n-Hexane, chloroform and water as solvent in succession. The obtained fractions were concentrated under reduced pressure to yield corresponding extracts. The obtained extracts and fractions were kept in a desiccator to remove moisture and stored properly until used. Yield of each extract and fraction is noted in terms of w/w of dry material.

3.1.3. STUDIES ON METHANOLIC EXTRACTS AND FRACTIONS OF *BM*

3.1.3.1. Acute toxicological studies

All the animals handling and experimentation were conducted in accordance with the prior approved guidelines from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and the experimental protocol was approved by Institutional Animal Ethical Committee (IAEC/05/UCPSc/KU/2016). Acute oral toxicity was performed as per the procedure described in chapter 2.2.3.1.

3.1.3. PHARMACOLOGICAL STUDIES

3.1.3.1. Grouping and induction of neurotoxicity

Neurotoxicity was induced by intra cerebroventricular (i.c.v.) injection of $A\beta_{(25-35)}$ peptide by identifying bregma point in the skull using stereotaxic apparatus (INCO, Ambala, India) as per the procedure described in chapter 2.2.3.2. Each animal was injected with 10µl which contain 10µg of β-amyloid peptide (Laursen and Belknap,1986).

Treatment of the animals for extracts as follows:

- Group I (Control)
- Group II (Negative Control)
- Group III – Standard drug treatment (Donepezil, 5 mg/kg)
- Group IV- 200 mg/kg of MEBM
- Group V- 400 mg/kg of BMME

Treatment of the animals for fractions as follows:

- Group I (Control)
- Group II (Negative Control)
- Group III – Standard drug treatment (Donepezil, 5 mg/kg)
- Group IV- 200 mg/kg of BME
- Group V- 400 mg/kg of BME
- Group VI- 200 mg/kg of BMH
- Group VII- 400 mg/kg of BMH
- Group VIII- 200 mg/kg of BMQ
- Group IX- 400 mg/kg of BMQ
- Group X- 200 mg/kg of BMC
- Group XI- 400 mg/kg of BMC

3.1.3.2. BEHAVIORAL STUDIES

3.1.3.2.1. Jumping box (conditioned avoidance test)

It is done by using medicraft jumping box. Box divided into 2 equal chambers by plexiglass partition, with a gate providing access to adjacent compartment through 14×17 cm space and follows as per the procedure described in chapter 2.2.3.3.1.

3.1.3.2.2. Rectangular Maze test

Assessment of memory was done using Medicraft rectangular maze as per the method described in chapter 2.2.3.3.2.

3.1.3.2.3. Y-maze test

The Y-maze task was used to measure the spatial working memory in mice and it was determined according to the method explained in chapter 2.2.3.3.3.

3.1.3.3. BIOCHEMICAL ESTIMATIONS

3.1.3.3.1. Acetylcholinesterase (AChE) enzyme determination

The levels of AChE in amnesia mice are estimated as per the method described in chapter 2.2.3.4.1.

3.1.3.3.2. Assay of glutathione peroxidase (GPx)

The levels of GPx in amnesia mice are estimated as per the method described in chapter 2.2.3.4.3.2.

3.1.3.3.3. Estimation of MDA

The levels of MDA in amnesia mice are estimated as per the method described in chapter 2.2.3.4.5.

3.1.3.3.4. MAO ASSAY

The MAO – A and B activity of MEBM and selected fractions (BME) were determined as per procedure described in chapter 2.2.3.4.2 with the following treatment:

- Group I (Control)
- Group II (Negative Control)
- Group III – Standard drug treatment (Donepezil, 5 mg/kg)
- Group IV- 200 mg/kg of MEBM
- Group V- 400 mg/kg of MEBM
- Group VI- 200 mg/kg of BME
- Group VII- 400 mg/kg of BME

3.1.3.3.5. ESTIMATION OF ANTIOXIDANT ENZYME

Estimation of free radical screening activity of MEBM and selected fraction (BME) is determined by performing assay of SOD, catalase and vitamin C as per procedure described in chapter 2.2.3.4.3 with the following treatment:

- Group I (Control)
- Group II (Negative Control)
- Group III – Standard drug treatment (Donepezil, 5 mg/kg)
- Group IV- 200 mg/kg of MEBM
- Group V- 400 mg/kg of MEBM
- Group VI- 200 mg/kg of BME
- Group VII- 400 mg/kg of BME

3.1.3.3.6. DPPH radical scavenging activity assay

The free radical scavenging activity of different fractions on 2, 2-di-phenyl-2-picrylhydrazyl (DPPH) radical was measured by reduction of DPPH to DPPHH (Di-phenyl picryl hydrazine). IC_{50} value was determined as the inhibitory concentration of extract that could scavenge 50% of the DPPH radicals. Ascorbic acid was used as positive reference.

$$\%\text{inhibition} = \frac{\text{Absorbance (cont)-absorbance (sam)}}{\text{Absorbance (cont)}} \times 100\%$$

4.1 STUDIES ON METHANOLIC EXTRACT AND FRATIONS OF *BUTEA MONOSPERMA*

4.1.1. Physical nature and percentage yield of extracts

The percentage yield and physical status of the methanolic leaf extract (MEBM) and its ethyl acetae (BME), n-Hexane (BMH), chloroform (BMC) and aqueous (BMQ) fractions *B. monosperma* are showed in the Table 4.1. Yield of the extracts calculated with reference of the weight raw material used. For fractions, the yield was calculated with respect to corresponding methanolic extract.

Table 4.1: Percentage yield and physical status of the methanolic extract and fractions of *B. monosperma*.

Extract/Fraction	Code	Yield (%)
Methanolic	MEBM	12.5
Ethyl acetate	BME	6.5
Chloroform	BMC	5.3
n-hexane	BMH	6.1
Aqueous	BMQ	3.3

4.1.2. ACUTE TOXICITY STUDIES

The MEBM and fractions were considered as nontoxic, because it did not show any toxic signs or symptoms and mortality in the oral dose of 2000 mg/kg in mice. According to OECD-423 guidelines, the LD_{50} of 2000 mg/kg and above is mentioned as unclassified. So further pharmacological screening is carried out. Hence, two doses (1/10th and 1/5th of 2000 mg) methanolic extracts and their fractions were selected for the neuroprotective study.

4.1.3. *IN-VIVO* PHARMACOLOGICAL ACTIVITY

The Stereotaxic apparatus used to identify the bregma point over the skull (approximately 1-3 mm rostral to the line drawn through anterior base of ears). After the identification, amnesia in mice was induced by $A\beta_{(25-35)}$ by i.c.v. injection and the mice were grouping for the treatment after the induction. The animals were subjected to behavioral, biochemical and free radical scavenging activity.

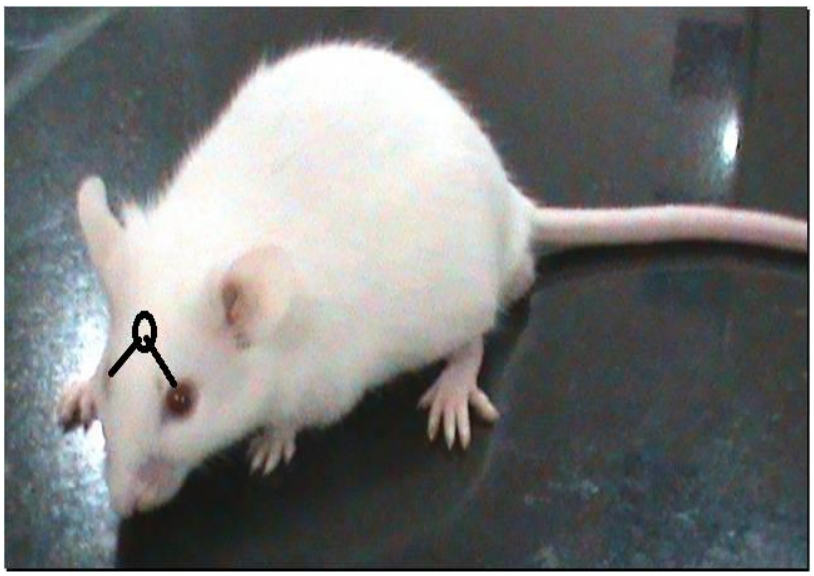

Figure 4.1: Identification of bregma point by Steriotaxic apparatus.

Bhevariouals studies

In behavioral studies, the methanolic extract of BM and its fractions exhibited improvement in cognitive function associated with changes of memory and behavior in amnesia induced mice. The results of different behavioral tests (jumping box, rectangular and Y- maze test) are showed in Table 4.2 and 4.3.

Jumping box test

The jumping box test also considered as conditioned avoidance test (memory). The activity was expressed in latency periods with sec time in amnesia induced mice. In jumping box test, there was an in increase in latency period in negative control group (24.6±2.07) when compared to vehicle control (12.5 ± 0.22) (Table 4.2 and Figure 4.2). The MEBM treated group showed decrease in latency period in groups treated (19.5±0.67 and 14 ± 1.09 for 200 and 400 mg/kg).

Table 4.2: Effect of MEBM and its fractions on behavioral activity by Jumping box, rectangular maze and Y-maze test (Mean±SD, n=6).

***BM* extract**			
Group	**Jumping box test (Sec)**	**Rectangular maze test (Sec)**	**Y-maze test (% alterations)**
I (PBS)	12.5 ± 0.22	47.83 ± 2.37	47.61 ± 3.15
II (Aβ)	24.6 ± 2.07	144.66 ± 10.05	25.57 ± 2.87
III (Aβ + DPZ)	15.69 ± 1.21***	71.66 ± 2.15***	43.93 ± 2.38***
IV (200mg/kg MEBM+ Aβ)	19.57± 0.71*	81.16 ± 2.21**	38.11±2.06**
V (400 mg/kg MEBM + Aβ)	14 ± 1.09***	76.87±2.53***	41.19 ± 2.18**

*p < 0.05, **P < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test.

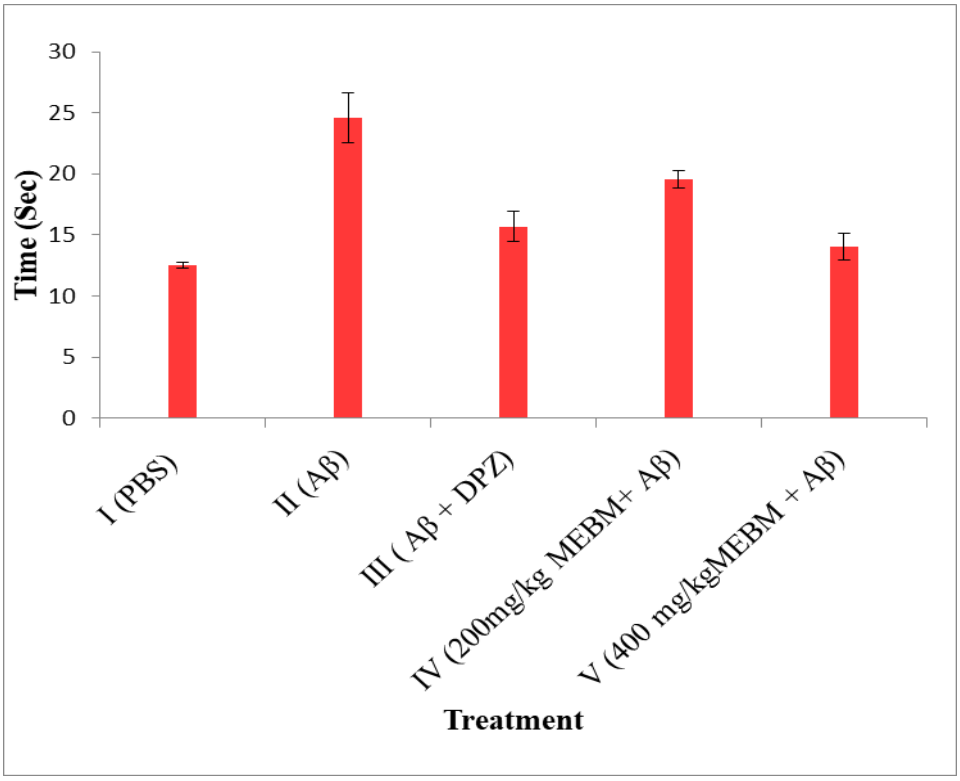

Figure 4.2: Effect of MEBM on jumping box test in amnesia mice (mean±SD, n=6).

In case of fractions treated groups, there is a decrease in latency period in groups treated with BME (17.4±1.09 and 13.1±1.17 for 200 and 400 mg/kg), BMC (19.7±1.10 and 15.3±0.75 for 200 and 400 mg/kg), BMH (18.1±0.62 and 14.4±0.71 for 200 and 400 mg/kg) and BMQ (20.8±1.24 and 16.3±1.17 for 200 and 400 mg/kg). From the results, the BME fraction shows statistically significant more activity compared to BMC, BMH and BMQ fractions and also all the fractions exhibited dose dependent effect (Table 4.3 and Figure 4.3).

Table 4.3: Effect of different fractions on behavioral activity by Jumping box, rectangular maze and Y-maze test.

Group	Jumping box test (Sec)	Rectangular maze test (Sec)	Y-maze test (% alterations)
I (PBS)	12.5 ± 0.22	47.83 ± 2.37	47.61 ± 3.15
II (Aβ)	24.6 ± 2.07	144.66 ± 10.05	25.57 ± 2.87
III (Aβ + DPZ)	15.69 ± 1.21***	71.66 ± 2.15***	43.93 ± 2.38***
IV (Aβ + 200mg/kg BME)	17.4 ± 1.09**	81.16 ± 2.21**	38.21±2.14**
V (Aβ + 400mg/kg BME)	13.1 ± 1.17***	71.55 ± 3.84***	43.77±2.84***
VI (Aβ + 200 mg/kg BMH)	18.1±0.62**	85.76±2.81**	34.26±1.32*
VII (Aβ + 400 mg/kg BMH)	14.4±0.71**	73.79±2.54***	41.81±2.47**
VI III (Aβ + 200mg/kg BMQ)	20.8±1.24*	105.6±3.74*	29.54±1.08
IX(Aβ + 400mg/kg BMQ)	16.3±1.17**	96.7±2.86*	37.76±1.79**
X (Aβ + 200mg/kg BMC)	19.7±1.10*	97.4±2.48*	32.75±1.93*
XI (Aβ + 400mg/kg BMC)	15.3±0.75**	88.3±3.26**	39.88±2.05**

*p < 0.05, **P < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test.

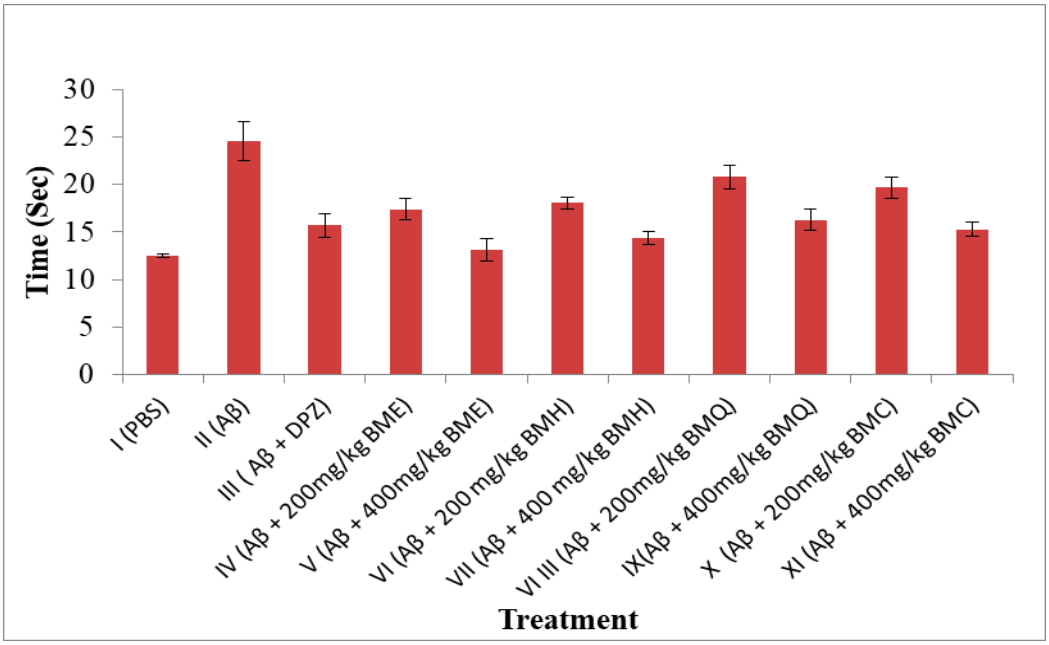

Figure 4.3: Effect of different fractions on jumping box test in amnesia mice (mean±SD, n=6).

Rectangular maze test

The hippocampal learning of Aβ induced group (negative control) was declined and shown a significant (p<0.001) increase in escape latency while comparing the control group. The readings were showed in Table 4.2 and Table 4.3. From the results, in rectangular maze test there was an increase in maze traverse period in negative control group (144.66±10.05) when compared to control (47.86±2.37), and there is a decrease in traverse period in groups treated

with methanol extract (81.16±2.21). The fractions treated groups also showed a dose dependent decrease in traverse period in BME (81.16 ± 2.21 and 71.55 ± 3.84), BMC (97.4±2.48 and 88.3±3.26), BMH (85.76±2.81 and 73.79±2.54) and BMQ (105.6±3.74 and 96.7±2.86) fractions. The results of test were represented in Figures 4.4 to 4.5 for MEBM and fractions of MEBM.

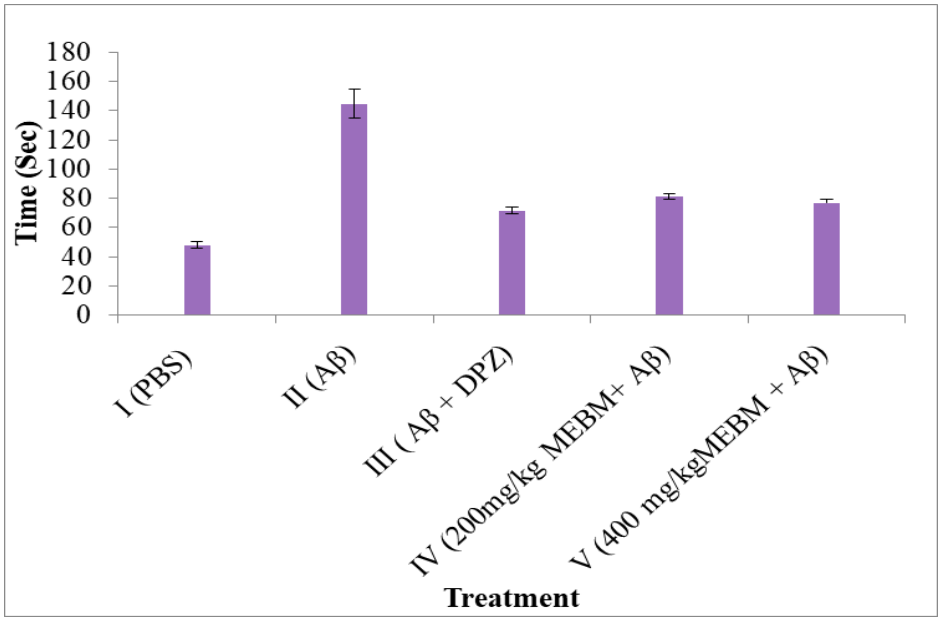

Figure 4.4: Effect of MEBM on rectangular maze test in amnesia mice (mean±SD, n=6).

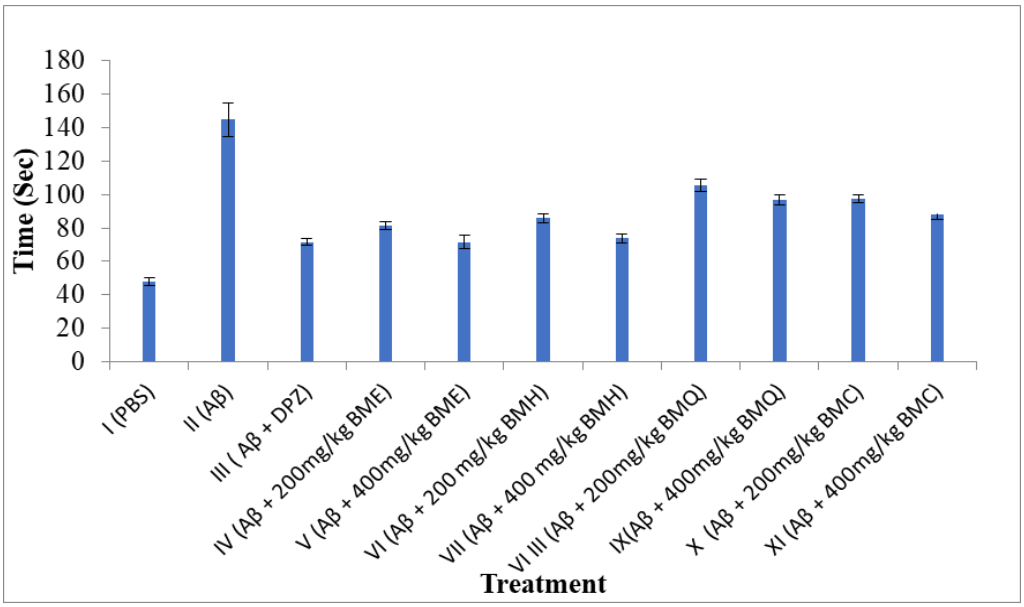

Figure 4.5: Effect of different fractions on rectangular maze test in amnesia mice (mean±SD, n=6).

Y-maze test

The Y-maze test was performed in amnesia mice and reported the outcomes in % alterations. The results showed in Table 4.2 and 4.3 and Figures 4.6 and 4.7. As the dose increased from 200 to 400 mg/kg, % alteration was increased. From the results, in negative control and vehicle groups the % alteration was found to be 25.57 ± 2.87 and 47.61 ± 3.15, respectively. MEBM showed 38.11±2.06 and 41.19 ± 2.18 % alterations for 200 and 400 mg/kg respectively,

which was statistically significant (p<0001) compared to negative control group. In case of BME, BMH, BMC and BMQ the % alteration were found to be 38.21±2.14 and 43.77±2.84, 34.26±1.32 and 41.81±2.47, 32.75±1.93 and 39.88±2.05; 29.54±1.08 and 37.76±1.79 for 200 and 400 mg/kg, respectively and was significantly (p<0.001).

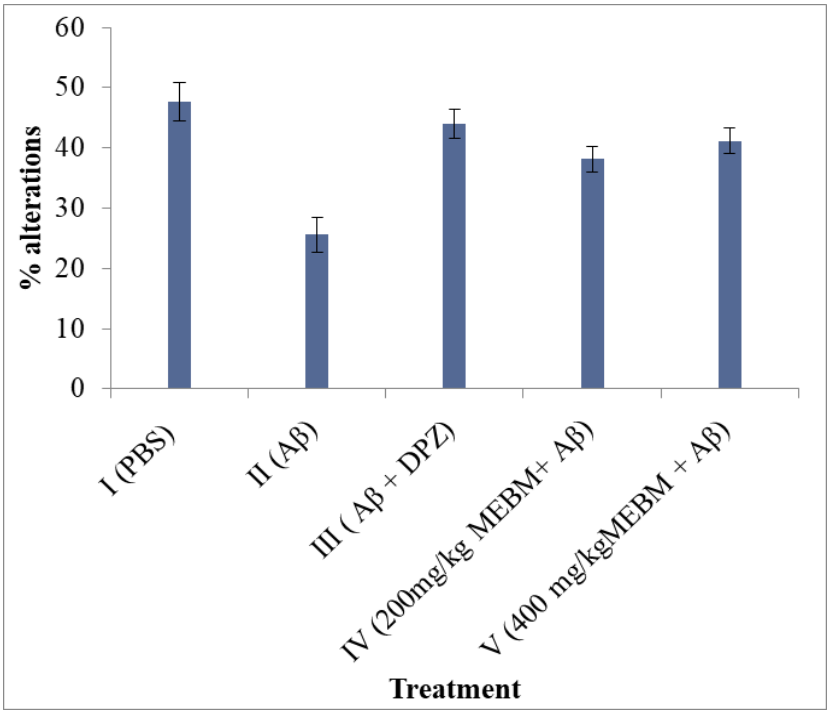

Figure 4.6: Effect of MEBM on Y-maze test in amnesia mice (mean±SD, n=6).

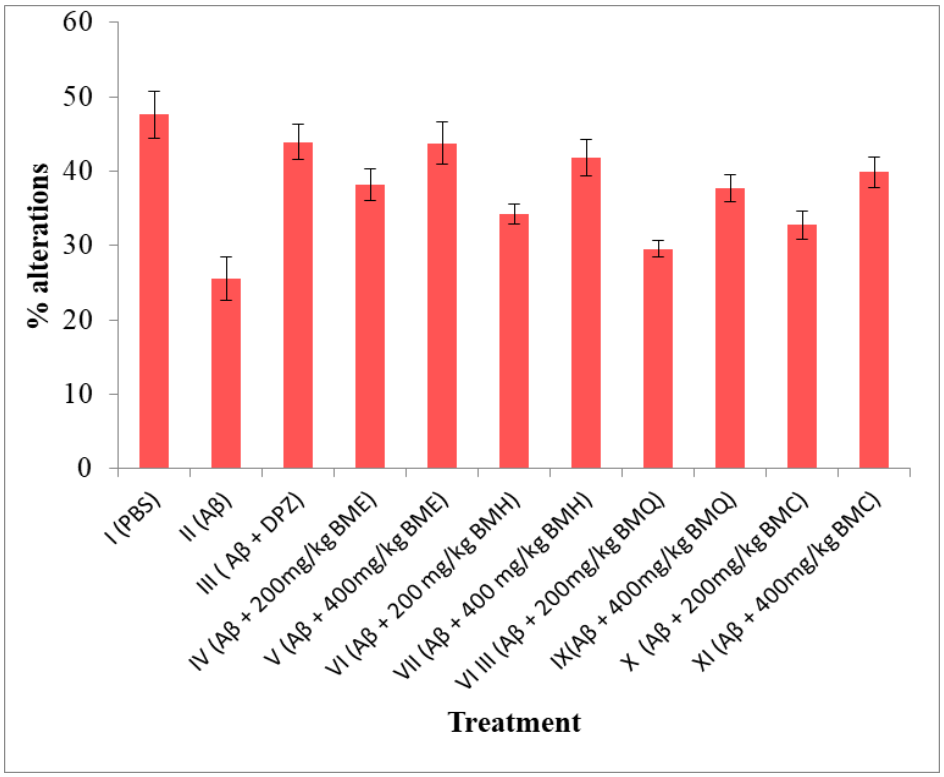

Figure 4.7: Effect of different fractions on Y-maze test in amnesia mice (mean±SD, n=6).

4.1.4. BIOCHEMICAL ESTIMATIONS

The MEBM and fractions (BME, BMH, BMC and BMQ) were subjected to various biochemical parameters i.e., AChE, GSH and MDA levels. The differences in biochemical parameters in treatment groups were summarized in the Table 4.4 and 4.5.

Acetylcholinesterase (AChE) enzyme

The i.c.v injection of Aβ peptide in negative control animals showed an extremely significant (P<0.001) increase in brain AChE levels. The AChE enzyme levels were significantly decreased in the treatment groups and indicated a difference with p<0.01, p<0.05, p<0.001 and p<0.05, respectively when compared with the amnesia induced group. The levels of each animal expressed in µmole/min/g.

From the results, it was found that acetyl cholinesterase levels are increased in negative (0.23±0.015) when compared to control (0.10±0.0012), and decreased levels are observed in methanol extract (0.15±0.0026 and 0.13 ±0.0031 for 200 and 400 mg/kg, respectively) (Figure 4.8). The AChE enzyme levels were significantly decreased in the treatment groups of BME (0.15 ± 0.0026 and 0.12 ± 0.0023), BMC (0.19 ± 0.0025 and 0.16± 0.007), BMH (0.17 ± 0.0021 and 0.13 ± 0.0018) and BMQ (0.21 ± 0.0046 and 0.18± 0.0051) and indicated a difference with p<0.01, p<0.05, p<0.001 and p<0.05, respectively when compared with the amnesia induced group (Figure 4.9). The decreased levels of AChE were increased along with dose of the extract as well as fractions.

Table 4.4: Effect of MEBM on biochemical parameters (mean±SD, n=6).

Group	AChE (µmole/min/g)	GSH (µmole/min/mg protein)	MDA (µg/gm wet tissue)
I (PBS)	0.10 ± 0.0012	0.0298 ± 0.00014	23.46 ± 0.30
II (Aβ)	0.23 ± 0.0015	0.0159 ± 0.00023	51.63 ± 0.54
III (Aβ +DPZ)	0.11 ± 0.0011***	0.0281 ± 0.0012***	26.66 ± 0.35***
IV (200mg/kg + Aβ)	0.15±0.0026**	0.0266±0.00186*	39.98±0.3012*
V (400 mg/kg + Aβ)	0.13 ± 0.0031***	0.0275± 0.00 14*	30.14 ± 1.42*

*****p < 0.05, **p < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test.

Table 4.5: Effect of different fractions of MEBM on different biochemical parameters (mean±SD, n=6).

Group	AChE (µmole/min/g)	GSH (µmole/min/mg protein)	MDA (µg/gm wet tissue)
I (PBS)	0.10 ± 0.0012	0.0298 ± 0.0014	23.46 ± 0.30
II (Aβ)	0.23 ± 0.0015	0.0159 ± 0.0023	51.63 ± 0.54
III (Aβ + Donepezil)	0.11 ± 0.0011***	0.0281 ± 0.0012***	26.66 ± 0.35***
IV (Aβ + 200mg/kg BME)	0.15 ± 0.0026**	0.0256 ± 0.0018**	39.98 ± 1.07**
V (Aβ + 400 mg/kg BME)	0.12 ± 0.0023***	0.0279 ± 0.0025***	31.26 ± 1.88***
VI(Aβ + 200mg/kg BMH)	0.17 ± 0.0021**	0.0247 ± 0.008**	41.36 ± 1.16*
VII (Aβ + 400mg/kg BMH)	0.13 ± 0.0018***	0.0268 ± 0.004***	34.78 ± 1.43**
VIII (β + 200 mg/kg BMQ)	0.21 ± 0.0046	0.0227±0.0011*	47.72±1.86*
IX (Aβ + 400 mg/kg BMQ)	0.18 ± 0.0051*	0.0242±0.0024**	39.92±2.54**
X (Aβ + 200mg/kg BMC)	0.19 ± 0.0025*	0.0239±0.0013*	43.31 ± 1.62*
XI (Aβ + 400mg/kg BMC)	0.16 ± 0.007**	0.0253±0.0027**	33.67 ± 2.64**

*****p < 0.05, **p < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test

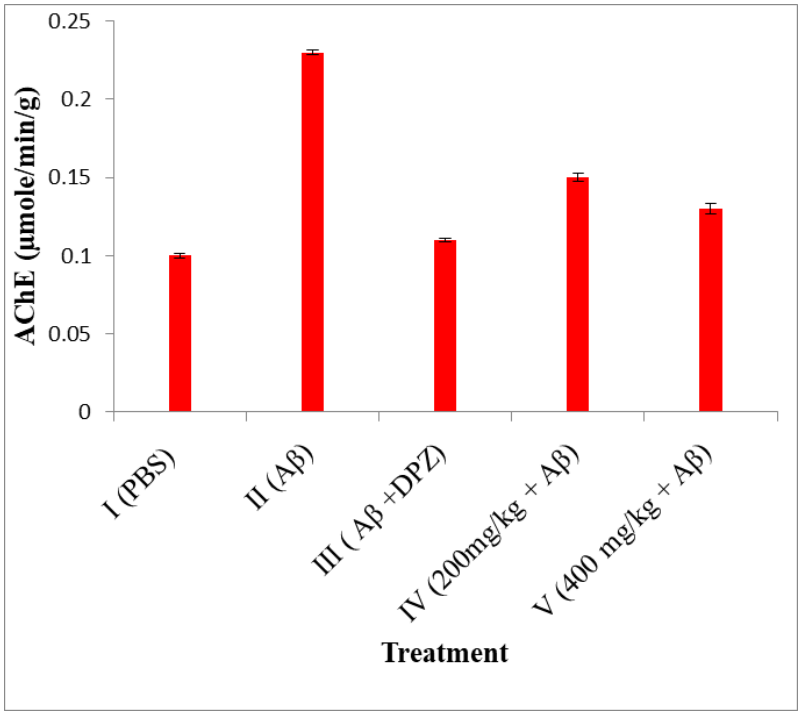

Figure 4.8: Effect of MEBM on AChE levels in amnesia mice (mean±SD, n=6).

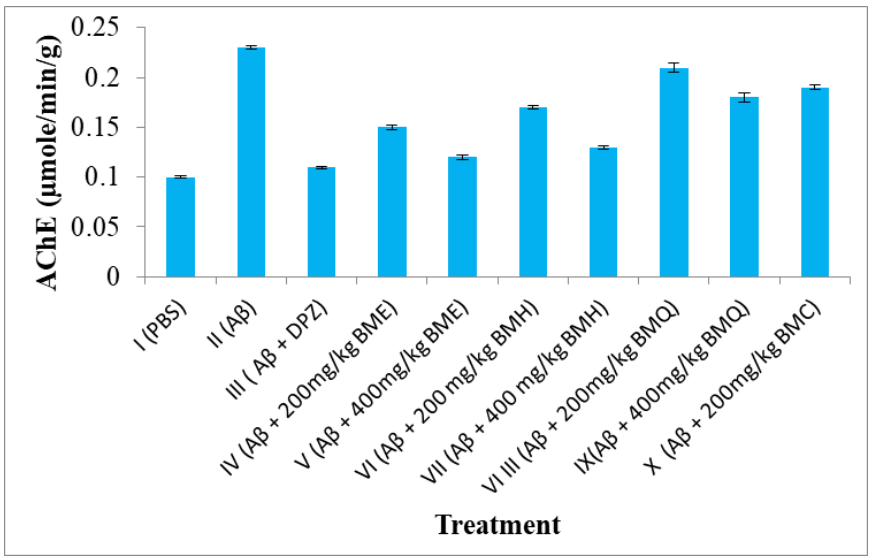

Figure 4.9: Effect of different fractions of MEBM on AChE levels in amnesia mice (mean±SD, n=6).

Glutathione peroxidase (GPx)

Glutathione levels are decreased in negative (0.0159±0.00023) when compared to control (0.0298±0.00014), and levels are increased in methanolic extract of 200 mg/kg and 400 mg/kg respictively as follows 0.0266±0.00186 and 0.0275± 0.00 14. Glutathione levels are decreased in negative (0.0159±0.00023) when compared to control (0.0298±0.00014), and levels are increased in BME (0.0256±0.0018 and 0.0279 ± 0.0025), BMC (0.0239±0.0013 and 0.0253±0.0027), BMH (0.0247 ± 0.0008 and 0.0268 ± 0.0004) and BMQ (0.0227±0.0011 and 0.0242±0.0024) fractions. The results are statistically significant compared with negative control ($p<0.05$). Figure 4.10 and 4.11 represented the effect of MEBM and its fractions on GSH levels.

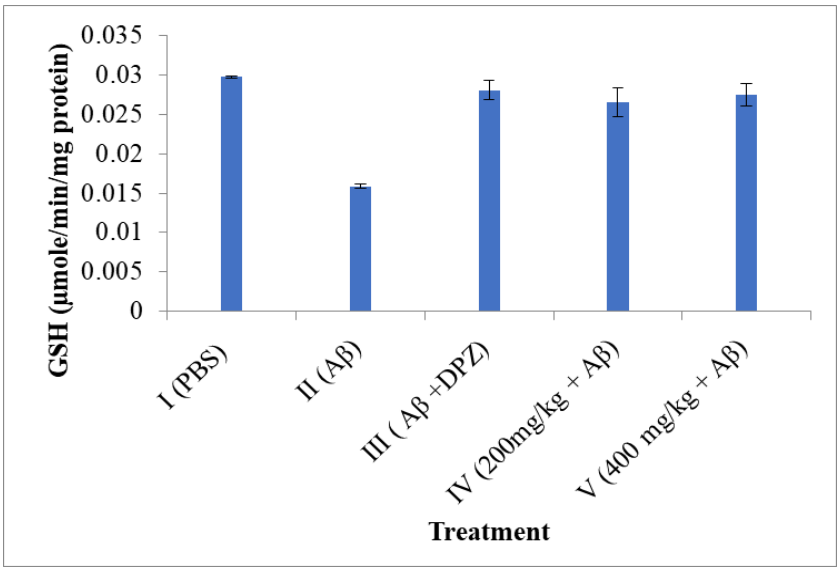

Figure 4.10: Effect of MEBM on GSH levels in amnesia mice (mean±SD, n=6).

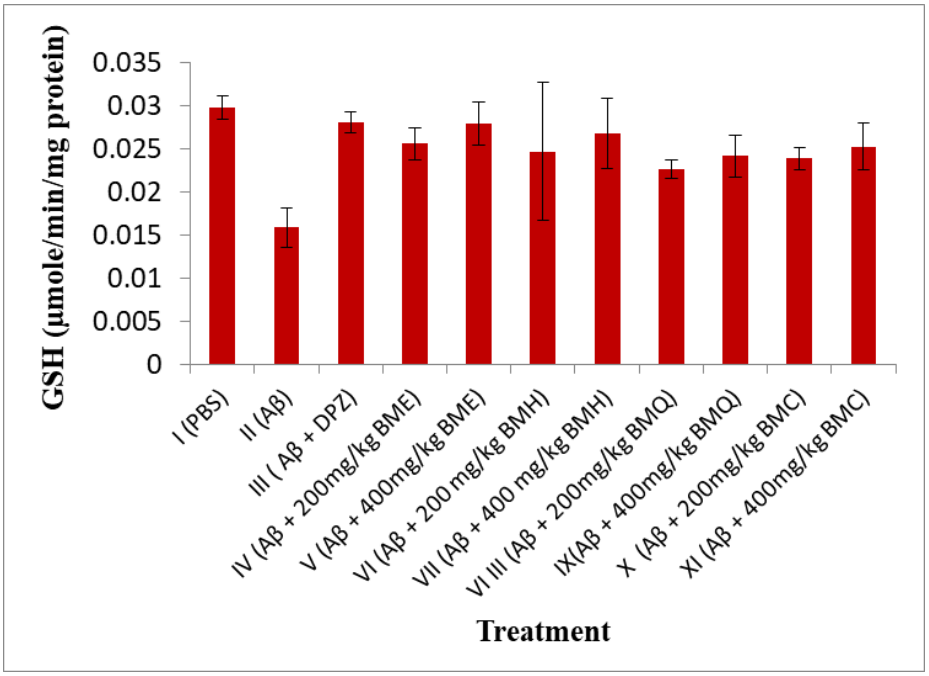

Figure 4.11: Effect of different fractions of BM on GSH levels in amnesia mice (mean±SD, n=6).

Estimation of MDA

MDA levels are increased in negative (51.63±0.5464) when compared to control (23.46±0.305), and decreased levels are observed in methanol extract was found to be 39.98±0.3012 and 30.14±1.42 µg/g/wet tissue for 200 and 400 mg/kg dose respectively. Decreased levels were observed in BME (39.98 ± 1.07 and 31.26 ± 1.88 for 200 and 400 mg/kg), BMC (43.31 ± 1.62 and 33.67 ± 2.64 200 and 400 mg/kg), BMH (41.36 ± 1.16 and 34.78 ± 1.43 200 and 400 mg/kg) and BMQ (47.72±1.86 and 39.92±2.54) fractions was observed. The decreased levels of MDA parameter levels were observed in fractions treated group and was the indication of anti-amnesic activity of BM fractions. The results are presented in Figure 4.12 and 4.13.

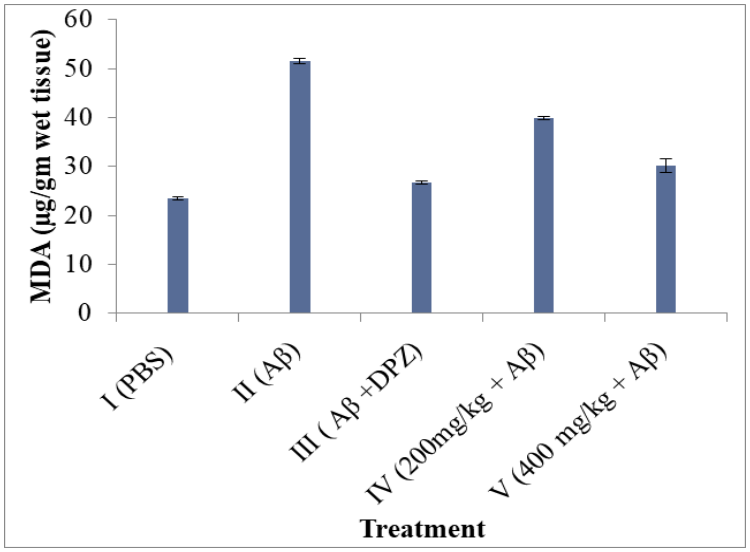

Figure 4.12: Effect of MEBM on MDA levels in amnesia mice (mean±SD, n=6).

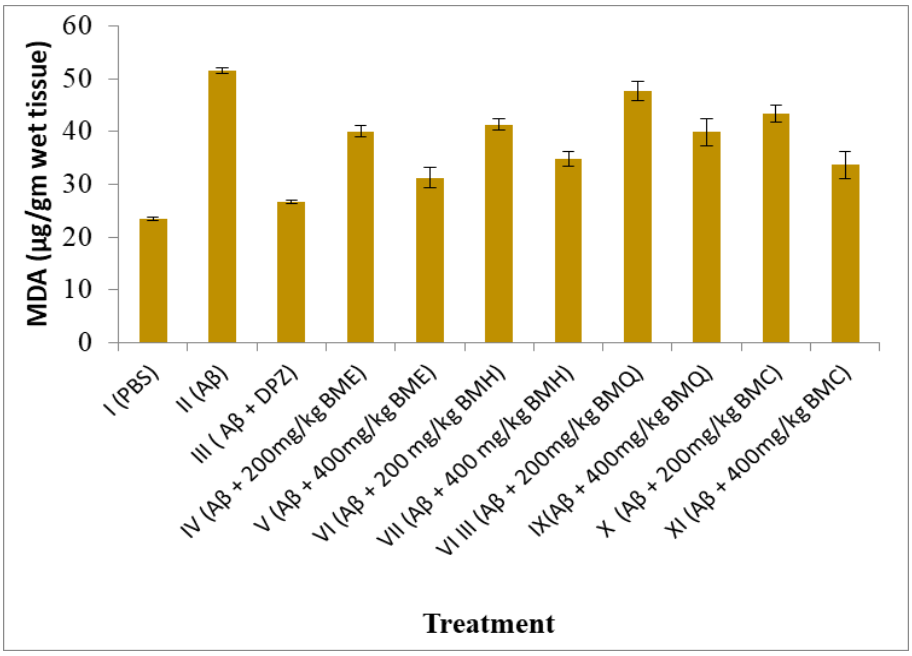

Figure 4.13: Effect of different fractions of MEBM on MDA levels in amnesia mice (mean±SD, n=6).

MAO ASSAY

The neurotransmitter metabolic enzyme responsible for the metabolism of bigenic amines in the brain were significantly (P<0.001) elevated after Aβ injection in negative control animals (31.88 ± 0.53 and 34.60±0.86 for MAO-A and B, respectively) when compared with the control group (20.32 ± 0.82 and 21.33±0.69 for MAO-A and B, respectively). In the treatment groups there was a significant reduction of MAO-A (P<0.001) and B (P<0.05) in low dose (200 mg/kg) treated animals on comparing to the control group. The high dose treated group shown a significant decrease in both MAO-A and B with P<0.001 on comparison with the neurotoxicity induced animals. Among the treated groups, the 400 mg/kg treatment showed a dose dependent significance (P<0.001). These actions of MEBM and BME on MAO-A and B were represented in Table 4.6 and Figures 4.14 and 4.15.

Table 4.6: Effect of MEBM and BME on MAO-A and B parameters (Mean ± SD, n=6).

Group	MAO (nmol/mg protein.h)	
	A	B
I (PBS)	20.32 ± 0.82	21.33±0.69
II (Aβ)	31.88 ± 0.53	34.60±0.86
III (Aβ + DPZ)	21.23 ± 0.66***	22.45±0.74***
IV (200mg/kg MEBM + Aβ)	26.56 ± 0.85**	31.37±0.59*
V (400 mg/kg MEBM + Aβ)	23.87 ± 0.66***	28.46±0.78**
VI (Aβ + 200 mg/kg BME)	25.45 ± 0.77**	29.05±0.94*
VII (Aβ + 400 mg/kg BME)	22.37 ± 0.89***	24.18±1.26**

*p < 0.05, **p < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test

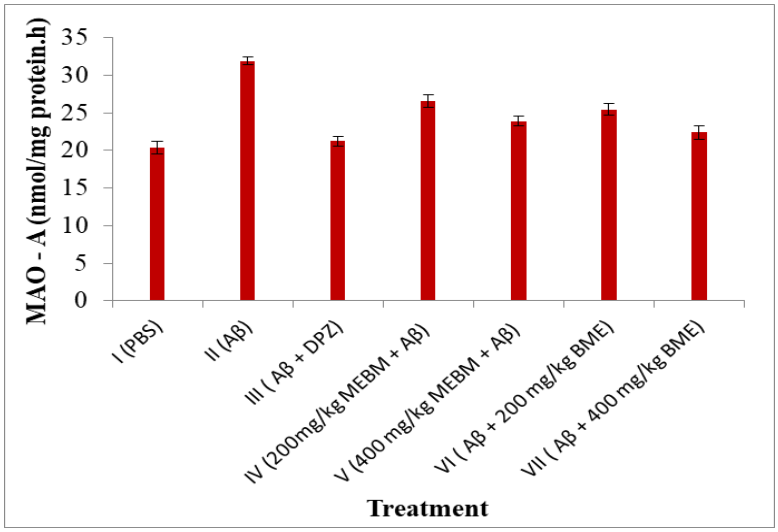

Figure 4.14: Effect of MEBM and BME on MAO-A levels in amnesia mice (mean±SD, n=6).

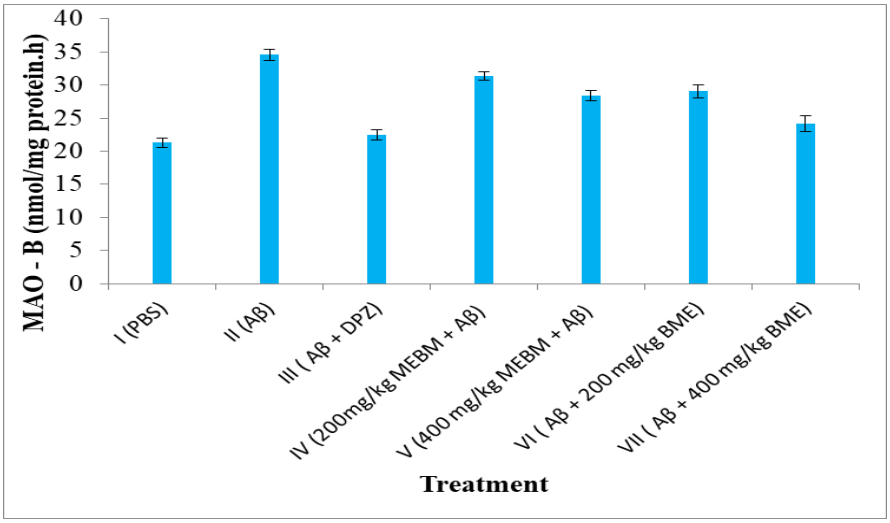

Figure 4.15: Effect of MEBM and BME on MAO-B levels in amnesia mice (mean±SD, n=6).

ESTIMATION OF ANTIOXIDANT ENZYME

The levels of SOD in the brain of Aβ induced group was significantly reduced (P<0.001) when compared to the phosphate buffered saline treated group. A significant (P<0.05 and P<0.001) escalation was exhibited in both low (200 mg/kg) and high dose (400 mg/kg) treated animals on comparison with amnesia induced group respectively. Among

the treatment groups, there was a significant (P<0.01) dose dependent increase in SOD was shown between 200 mg/kg dose and 400 mg/kg dose. In all the parameters of the antioxidant evaluation such as SOD, Catalase and Vitamin C, there was a significant (P<0.001) reduction in amnesia induced group when compared with that of the control group. The 200 mg/kg and 400 mg/kg treated animals significantly increased the levels of GPx with P<0.001 and P<0.01 respectively when compared with the negative control group. The dose dependent change of the GSH activity between the low dose and high dose indicated a significant (P<0.05) improvement.

In case of catalase enzyme the treatment of MEBM reversed the reduction with significance of P<0.05 in low dose and showed extremely significant (P<0.001) increase in high dose. When compared to the low dose treated group of animals, the high dose treated animals shown a significant (P<0.05) changes indicate the dose dependent activity of MEBM. The non-enzymic antioxidant, Vitamin C exhibited a significant increase of activity with P<0.01 and P<0.001 in low dose and high dose treated animals respectively on comparing with the neurotoxicity induced group. There is also a significant dose dependent activity in vitamin C was noted in 400 mg/kg treated group when compared to the 200 mg/kg treated group. All the antioxidant properties with various parameters were shown in Table 4.7 and Figures 4.16 to 4.19.

Table 4.7: Effect of MEBM and BME on free radical scavenging property.

Group	SOD (U/min/mg protein)	Catalase (U/mg protein)	VIT.C µg/mg protein.
I (PBS)	6.83 ± 0.57	2.17 ± 0.06	0.89 ± 0.07
II (Aβ)	1.73 ± 0.39	0.85 ± 0.09	0.51 ± 0.06
III (200 mg/kg MEBM+ Aβ)	4.26 ± 0.69*	1.57 ± 0.27*	0.67 ± 0.03*
IV(400 mg/kg MEBM+ Aβ)	5.94±0.77***	1.83 ± 0.24**	0.75 ± 0.03**
V (Aβ + 200 mg/kg BME)	4.54 ± 0.75**	1.81 ± 0.26**	0.71 ± 0.06**
VII (Aβ + 400 mg/kg BME)	6.15 ± 0.68***	2.04 ± 0.38***	0.82 ± 0.05***

*p < 0.05, **p < 0.01, ***p < 0.001, when compared to negative control. ANOVA (one-way) followed by Bonferroni's test.

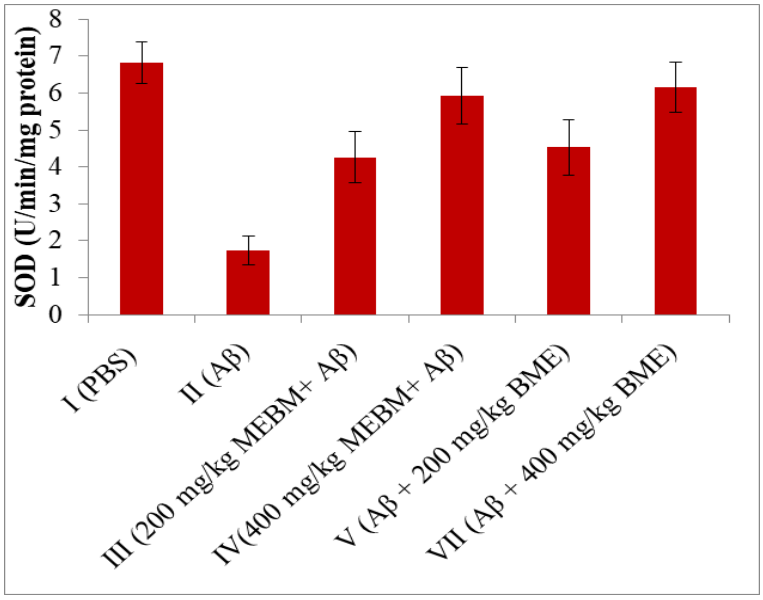

Figure 4.16: Effect of MEBM and BME on SOD levels in amnesia mice (mean±SD, n=6).

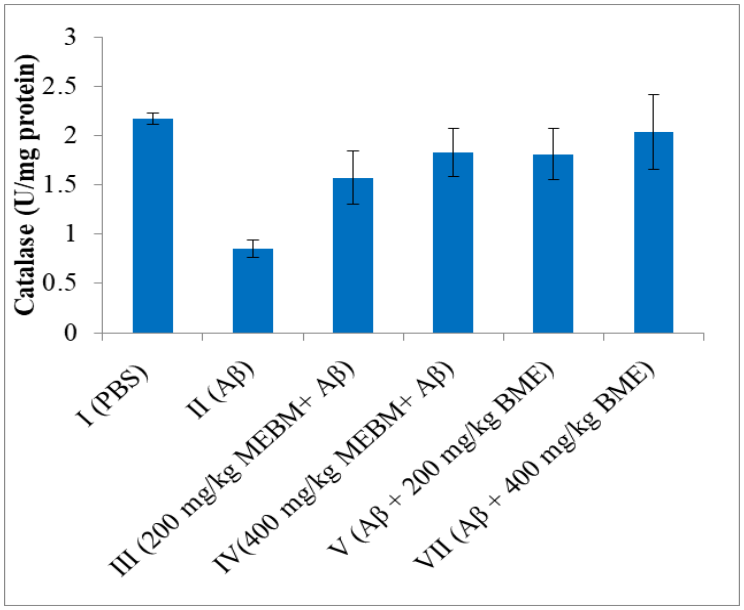

Figure 4.17: Effect of MEBM and BME on Catalase levels in amnesia mice (mean±SD, n=6).

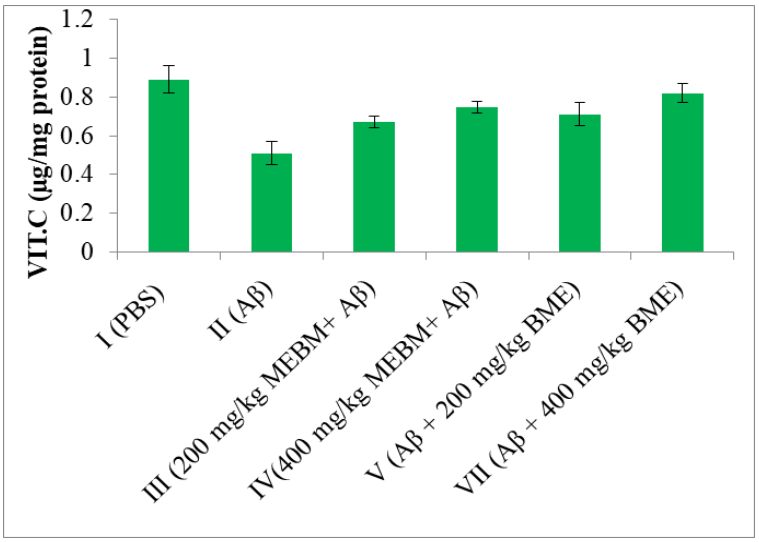

Figure 4.18: Effect of MEBM and BME on VIT.C levels in amnesia mice (mean±SD, n=6).

The antioxidant property of different MEBM and fractions were evaluated by using DPPH free radical scavenging assay and these fractions exhibited dose dependent free radical scavenging activity. The calibration curve of ascorbic acid was plotted in the linearity range of 1-10 nM/mL. The correlation coefficient was 0.999 (Table 4.8 and Figure 4.19). The IC_{50} value of MEBM, BME, BMC, BMH and BMQ fractions were found to be 29.76, 26.42, 33.77, 30.15 and 41.04 µg/mL, with respective to IC_{50} value of ascorbic acid and was found to be 12.92 µg/mL.

Table 4.8: Standard graph of ascorbic acid.

Concentration (nM/mL)	Absorbance
1	0.124
2	0.233
4	0.442
6	0.626
8	0.82
10	0.998

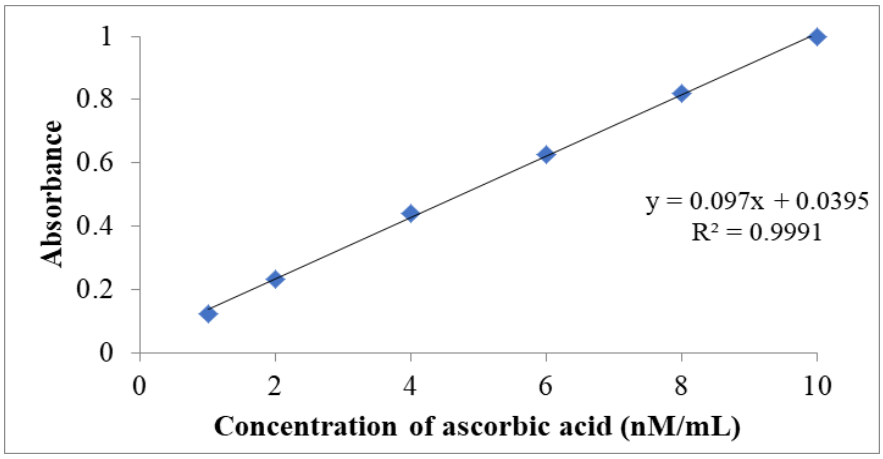

Figure 4.19: Calibration curve graph of ascorbic acid.

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