SCIENTIFIC EVALUATION OF BRAHMA RASAYANA AS A COGNITIVE ENHANCER IN D-GALACTOSE INDUCED COGNITIVE IMPAIRMENT IN MICE

A dissertation submitted to
THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY
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in partial fulfilment of the requirements for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACOLOGY

Submitted by
Reg. No. **261426067**

Under the guidance of
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CERTIFICATE

This is to certify that the dissertation entitled "**SCIENTIFIC EVALUATION OF BRAHMA RASAYANA AS A COGNITIVE ENHANCER IN D-GALACTOSE INDUCED COGNITIVE IMPAIRMENT IN MICE"** submitted by the **Reg. No. 261426067** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by the Tamilnadu Dr.M.G.R Medical University, Chennai is a bonafide work done by him during the academic year 2015-2016 under the guidance of **Dr. N. Jayshree, M.Pharm., Ph.D.,** Professor in Pharmacology, Institute of Pharmacology, Madras Medical College, Chennai-03.

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INDEX

LIST OF ABBREVIATIONS

AD	-	Alzheimer's disease
CSHA	-	Canadian Study of Health and Aging
CI	-	Cognitively impaired
NCI	-	Non cognitively impaired
nAChRs	-	Nicotinic acetylcholine receptors
AChE	-	Acetylcholinesterase
mGlu5	-	metabotropic glutamate receptor subtype 5
CNS	-	Central nervous system
DA	-	Dopamine
5-HT_6	-	5-hydroxytryptamine receptor 6
D_1	-	Dopamine receptor type 1
PFC	-	Prefrontal cortex
PD	-	Parkinson's disease
NMDA	-	N-methyl-D-aspartate
BR	-	Brahma Rasayana
IFN-γ	-	Interferon-gamma
IL-2	-	Interleukin-2
GM-CSF	-	Granulocyte macrophage-colony stimulating factor
CDDP	-	Cis dichloro diamine platinum II
CT	-	Clitoria ternatea
PTZ	-	Pentylenetetrazol
MES	-	Maximal electroshock
DG	-	Desmodium gangeticum
CA	-	Centella asiatica
TC	-	Terminalia chebula
DMBA	-	7,12-dimethylbenz[a] anthracene

$AlCl_3$	-	Aluminium chloride
Pb	-	Lead
IAEC	-	Institutional Animal Ethics Committee
CPCSEA	-	Committee for the purpose of control and supervision of experiments on animals
s.c	-	subcutaneous
GABA	-	Gamma amino butyric acid
DTNB	-	5,5’-dithio-bis-2-nitrobenzoic acid
HCL	-	Hydrochloric acid
rpm	-	Revolutions per minute
OPT	-	O- phthaladehyde
EDTA	-	Ethylenediaminetetraacetic acid
Na_2So_3	-	Sodium sulphite
$CuSo_4$	-	Copper sulphate
SEM	-	Standard error mean
ANOVA	-	Analysis of variance
EL	-	Escape latency
Ach	-	Acetylcholine

INTRODUCTION

Cognition refers to thinking skills and the intellectual skills that allow a person to perceive, acquire, understand and respond to information. This includes the abilities to pay attention, remember and process information, solve problems, organize and reorganize information, communicate and act upon information.[1]

The disorders that affect cognition include neurodegenerative disorders, vascular diseases, trauma, toxin, anoxia and infectious processes. Cognitive impairments are deficits in the processes by which persons perceive, encode, store, retrieve and use information. Many processes can lead to cognitive impairment. Neurodegenerative disorders that can affect cognitive ability include Alzheimer's disease (AD), Pick's disease, Parkinson's disease (PD), Lewy body disease, Huntington's disease, progressive supranuclear palsy and cerebellar degeneration. Common vascular disorders that affect cognition include stroke, multiple strokes and cerebral embolic disease. Toxic agents that affect cerebral function (cognition) include carbon monoxide poisoning, adverse drug events and interactions and prolonged exposure to specific toxic chemicals or gases. Electrolyte abnormalities, organ failure and anoxia can also cause cognitive impairments.[2]

There are two approaches to defining cognitive decline with age: 1) that which is considered part of the normal aging process and 2) that which is associated with underlying pathology and considered an atypical or abnormal aging process. From the first perspective, cognitive decline has been described as a natural and normal process experienced by the aged. From the second perspective, cognitive decline is viewed as a pathological process. Cognitive decline has also been associated with decreased cortical glucose metabolism, heightened protein levels (phosphorylated tau) in the cerebral spinal fluid and medial temporal lobe atrophy.[3]

Depression is the most prominent neuropsychiatric condition associated with cognitive impairment. In a study using data from the Canadian Study of Health and Aging (CSHA), higher rates of proxy-reported depression, loss of interest and changes in personality and mood were noted among cognitively impaired (CI) persons, compared to those who were not cognitively impaired (NCI).[3]

Role of various neurotransmitters in the cognitive enhancement

Aging is one of the most significant risk factors for Alzheimer's disease (AD). When the cognitive functions declines due to aging, it is called as cognitive aging. Cognitive function begins to decline in young adulthood, possibly as early as in the second or third decade of life. The development of novel cognitive enhancing therapies is important for improving function and quality of life for individuals who are suffering from cognitive impairment due to cognitive aging or AD.[4]

Acetylcholine

The neuronal nicotinic acetylcholine receptors (nAChR) is one of the important targets for cognitive enhancing therapies. The nAChRs are expressed in the hippocampus, a key brain area implicated in cognitive dysfunction in both aging and AD. The nAChRs function both presynaptically to regulate neurotransmitter release and postsynaptically where they activate intracellular signaling cascades involved in learning and memory.

In addition to nicotinic agonists, M_1 muscarinic agonists have the potential to boost cognition and slow disease progression. Post synaptic M_1 muscarinic Acetylcholine (Ach) receptors play a major role in hippocampal based memory and learning.[4]

Glutamate

Glutamate is the major excitatory neurotransmitter and is involved in almost all CNS functions, especially in cortical and hippocampal regions. About 70% of all excitatory synapses in the CNS utilize Glutamate as neurotransmitter, consistent with the involvement of the glutamatergic system in learning and memory. The disturbance in Glutamate neurotransmission leads to pathophysiological changes in AD. Chronic, mild activation of NMDA receptors ultimately leads to neurodegeneration and this effect is termed as chronic 'Excitotoxicity'. The prolonged ca^{2+} overload leads to loss of synaptic function followed by synaptotoxicity and ultimately cell death. It correlates with the loss of memory function and learning ability in AD patients.[5]

Glutamate can modulate excitatory postsynaptic currents via the metabotropic glutamate receptor subtype 5 (mGlu5). Increasing the activation of mGlu5 may offer an exciting new therapeutic enhance cognitive function in AD patients. Jeff Conn's group at Vanderbilt University has developed a series of mGlu5 positive allosteric modulators for AD, schizophrenia and other neurological conditions.[4]

Serotonin

In contrast to direct cholinergic stimulation, another potential therapeutic avenue is indirect cholinergic stimulation through 5-hydroxytryptamine ($5\text{-}HT_6$) receptors. Evidence from cellular and animal models suggest that $5\text{-}HT_6$ receptors may enhance cholinergic signaling through glutamatergic and gamma-amino butyric acidergic pathways. Both agonist and antagonist ligands of this poorly understood G-protein coupled recptor have been shown to enhance cognition in preclinical rodent models. Several ligands currently in early-phase clinical trials show promise as a symptomatic treatment for AD and are also being evaluated in combination with AChE inhibitors.[4]

Dopamine

Regarding cognitive functions, inactivation of the D_1 receptor gene produce spatial learning deficits which can be related to the involvement of the D_1 receptors in working memory processes in the prefrontal cortex (PFC) and to the presence of such receptors in the hippocampus. Thus, studies on these receptors should be relevant for further characterization of the involvement of Dopamine (DA) in the regulation of cognitive processes, as indicated by pharmacological studies.

The integrative properties of the dopaminergic system are probably associated more with direct contributions to cognitive functions at the cortical level, namely in working memory, executive functions and possibly time estimation processes. Because cognitive impairments occur early in PD, it has been suggested that even partial DA depletion limited to the striatal area could contribute to the neuropsychological symptoms.

Since dopaminergic brain activity apparently decreases with normal aging, correlated impairment in behavior, such as lack of flexibility and adaptive capacities, deficits in selective attention processes or working memory and executive function deficiencies, may be related to impairment of central dopaminergic transmission. Consequently, stimulating dopaminergic transmission in the elderly could represent a reliable strategy for improving behavioral deficits.[6]

Nor-adrenaline

Modulation of noradrenergic system has the potential to be both a symptomatic and disease modifying therapeutic strategy. The noradrenergic neurons of the locas coeruleus exhibit selective vulnerability during aging, with a significant loss of these cells and a decrease in noradrenaline observed in mild cognitive

impairment and AD patients. Noradrenaline is an excitatory neurotransmitter that also has anti-inflammatory properties. Regulators of noradrenaline levels are clinically approved for use in neuropsychiatric disorders.[4]

Adrenaline

Adrenaline can produce retrograde enhancement of long term memory in humans. An endogenous adrenaline can modulate memory consolidation of the events, ensuring memory strength that is proportional to memory importance. There is evidence which suggests that adrenaline does have a role in long term stress adaptation and emotional memory. **S**tudies have also found that recognition memory involving adrenaline depends on the mechanism of β-adrenoreceptors.[7]

Though cognition impairment remains a major problem, there are only a few drugs available to treat the condition. The drugs available are,

a) Cholinergic activators- Tacrine, Rivastigmine, Donepezil, Galantamine

b) Glutamate (NMDA) antagonist – Memantine

c) Miscellaneous drugs – Piracetam, Pyritinol, Piribedil[8]

There are several plants with cognition enhancing and memory improving properties. They are, *Ginko biloba, Hesperia serrate, Ephedra sinensis, Centella asiatica, Acorus calamus, Bacopa monnieri, Withania somnifera* and *Ginseng.*[9]

Ayurveda uses single drug or formulations in age related cognitive decline. The drugs mentioned as Meddhya rasayanas specifically and others having medhya activity can be potentially used for prevention and management of age related cognitive decline. Plants like *Centella asiatica, Tinospora carfdifolia, Glycyrrhiza glabra* are known as Medhya rasayanas which directly promote cognition. Others

such as Brahmi *(Bacopa monnieri*) and Jyotishmati (*Celastrus panniculatus*) are known for memory improvement.[10]

Rasayana is one the eight clinical specialties of classical Ayurveda. Rasayanas replenish vital fluids of human body, thus keeping us away from diseases. They enhance qualities of rasa, enrich it with nutrients, so that one can attain longevity, memory, intelligence, youthfulness, complexion & voice, optimum development of physique. Rasayanas are rejuvenators, nutritional supplements and possess strong antioxidant activity. Some important Rasayanas are Triphala rasayana, Shilajit rasayana, Brahma rasayana and Chyawanprash. The cognition enhancing activity of the Rasayanas has also been proved scientifically.[11]

In a recent work, chyawanprash was studied for its beneficial effects on cognition in aged animals. It was proved to have memory enhancing property by virtue of its antioxidant effect, procholinergic action, increase learning ability and increase retention capacity.[12]

This study attempts to validate the cognition enhancing property of one of the commonly used Ayurvedic formulation Brahma Rasayana (BR).

REVIEW OF LITERATURE

Reviews related to Brahma Rasayana

Praveen Kumar *et al*., carried out a study on rasayanas including Brahma Rasayana. The rasayanas enhanced the humoral immune response as seen from the increased number of antibody forming cells and circulating antibody titre. These results indicate the usefulness of rasayanas as immunostimulating agent.[13]

Praveen Kumar *et al*., studied the effect of various rasayanas including Brahma Rasayana on tumour bearing mice. Administration of rasayanas had been found to enhance the natural killer cell activity in normal as well as in tumour bearing animals. Brahma Rasayana was found to have the maximum activity. Brahma Rasayana and Ashwagandha rasayanas were found to activate antibody-dependent cellular cytotoxicity significantly. All the rasayanas were found to stimulate antibody dependent compliment mediated tumour cell lysis. The results of these studies indicate the usefulness of rasayanas for immunostimulation in normal and diseased state. [14]

Rekha P S *et al*., reported that 10 and 50 mg/kg dose of Brahma Rasayana when given orally showed a significant increase in total leukocyte count and percentage of polymorphonuclear cells in irradiated mice. Bone marrow cellularity and alpha-esterase positive cells also increased significantly in radiation treated animals after Brahma rasayana administration. They also found that, enhanced serum levels of interferon-gamma [IFN-γ] interleukin-2 [IL-2] and granulocyte macrophage-colony stimulating factor [GM-CSF] in normal and irradiated mice. Therefore, proliferation of stem cells induced by Brahma rasayana in irradiated mice might be related to the stimulation of cytokine production.[15]

Rekha P S *et al*., carried out the study on Brahma Rasayana and reported that Brahma Rasayana could reduce the oxygen radicals and subsequently reduce the harmful effects produced by the oxygen free radicals mediated injuries.[16]

Thangapazham R L *et al*., reported that methanolic extract of Brahma Rasayana inhibited the proliferation, tube formation, cell migration in a dose dependent manner. Their study suggested the possible mechanism(s) of action of Brahma rasayana in the reduction of tumour growth and metastatic spread.[17]

K P Guruprasad *et al*., carried out the chromosomal aberrations and sperm abnormality study on Brahma Rasayana in male Swiss albino mice. They suggested that Brahma Rasayana does not elicit genotoxic effects in the mice. It was found to be a moderate enhancer of reproductive and mitotic cellularity and a protector against certain kinds of sperm abnormality.[18]

Aditya Menon *et al*., evaluated the nephroprotective effect of Brahma Rasayana using cisplatin induced nephrotoxicity in mice. Administration of Brahma Rasayana resulted in the prevention of nephrotoxic effect of CDDP (cis dichloro diamine platinumII). They concluded that Brahma Rasayana can be used to prevent the side effects of chemotherapeutic drugs.[19]

Reviews related to key ingredients of Brahma Rasayana

Jain NN *et al*., carried out a study on methanolic extract of *Clitoria ternatea* (CT) on cognitive behavior, anxiety, depression, stress and convulsions induced by Pentylenetetrazol (PTZ) and Maximal electroshock (MES). They reported that methanolic extract of CT possess nootropic, anxiolytic, antidepressant, anticonvulsant and antistress activity.[20]

Hanumanthachar Joshi *et al.,* carried out a study on *Desmodium gangeticum* (DG) to evaluate the antiamnesic effects in mice. They reported that pretreatment with aqueous extract of DG in a dose of 50, 100 and 200mg/kg p.o for seven successive days, significantly improved learning and memory in mice and reversed the amnesia induced by both scopolamine (0.4mg/kg, i.p) and natural ageing. They also suggested that DG decreased whole brain acetylcholinesterase activity.[21]

Anil Kumar *et al.,* reported the protective effect of aqueous extract of *Centella asiatica* (CA) against D-Galactose induced behavioral, biochemical and mitochondrial dysfunctions in mice. They also reported that animals treated with aqueous extract of CA showed significant improvement in behavior alterations, oxidative damage and mitochondrial enzyme complex activities.[22]

Ferial majed *et al.,* reported that topical application of 30mg/kg of *Terminalia chebula* (TC) possesses anticarcinogenic effect and is an effective suppressor of oxidative stress against DMBA (7,12-dimethylbenz[a] anthracene) /croton oil induced cutaneous damage. They also suggested that TC could be used for the prevention of skin cancer.[23]

Reviews related to induce cognitive and memory impairment

There are certain models available to induce the memory and cognitive impairments in laboratory animals. They are,

- β – Amyloid model[24,25]
- D-Galactose and $AlCl_3$ model[26-28]
- D-Galactose model[22, 29-31]
- Lead (Pb) model[32,33]

D-Galactose model is a chronic exposure model. It has been used for the evaluation of memory and cognitive enhancement properties of various plant extracts like *Centella asiatica*,[22] *Ginkgo biloba*,[34] *Curcuma longa*,[35] *Artemisia annua L*[36]., *Capparis spinosa L.*[37]

AIM AND OBJECTIVE

Brahma Rasayana is an Ayurvedic formulation, which contains several herbal constituents and is marketed for the treatment of drowsiness, fatigue, mental weakness, progeriasis, aging and disturbed memory.[38]

Literature review indicates that no specific study has been carried out to evaluate the cognition enhancement property of Brahma Rasayana. Some of the ingredients used in Brahma Rasayana have been tested for their cognition enhancing property.[20-23] One of the commonly used models to evaluate cognitive impairment is D-Galactose model.

The aim of this study is

- Evaluation of *in vivo* cognition enhancing potential of Brahma Rasayana using D-Galactose induced memory impaired mice.
- To elucidate the effect of Brahma Rasayana on various neurotransmitters associated with cognitive impairment.

DRUG PROFILE

Brahma Rasayana

Ayurvedic system of medicine contains many herbal products for natural rejuvenation. Among those products, Brahma Rasayana (BR) is one of the most valuable natural remedy for stress and tiredness. This herbal recipe has been prescribed by Lord Brahma. It rejuvenates the body and fights against tiredness, fatigue, early grey hairs and wrinkling (Skin rejuvenation and hair rejuvenation). It is claimed to be the best anti-aging formula. It also improves intelligence, memory and immune power.[38] Brahma Rasayana is mentioned in "The Ayurvedic Formulary of India", for the treatment of drowsiness, fatigue/lethargy, tiredness without exertion/ languor, mental weakness, senility/ progeriasis, aging and disturbed memory.[39]

The total list of ingredients of Brahma Rasayana are,[38]

- Pathya – Chebulic Myrobalan fruit rind – *Terminalia chebula*
- Amalaki – Indian gooseberry fruit – *Emblica officinalis Gaertn.*
- Bilwa – Bael (root) – *Aegle marmelos*
- Shyonaka – *Oroxylum indicum*
- Gambhari – Coomb Teak (root) – *Gmelina arboera*
- Patala – Trumpet (root) – *Stereospermum suaveolens*
- Agnimantha – *Premna corymbosa (Burm.f) Merr*
- Shalaparni – *Desmodium gangeticum*
- Prishniparni – *Uraria picta*
- Brihati – Indian Nightshade (root) – *Solanum indicum*

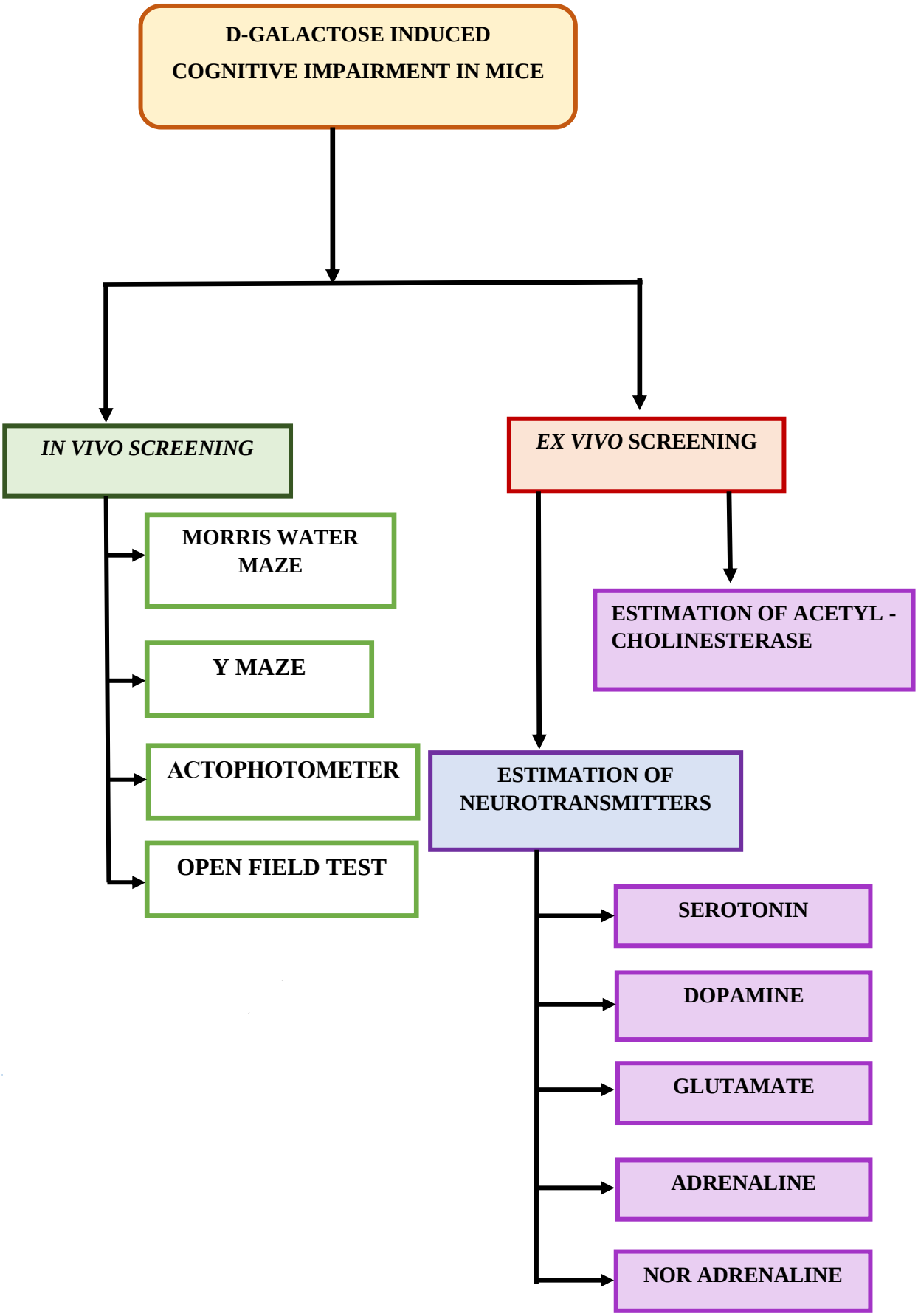
- Kantakari – Yellow berried nightshade (whole plant) – *Solanum xanthcarpum*
- Bala – Country mallow (root) – *Sida cordifolia*
- Punarnava – Spreading Hogweed – *Boerhaavia diffusa*
- Eranda – Castor – *Ricinus communis*
- Mashaparni – *Teramnus labialis / Vigna radiata*
- Mudgaparni – Green gram – *Phaseolus trilobus*
- Shatavari – *Asparagus racemosus* root
- Meda – *Litsea monopetala*
- Jivanti – *Leptadenia reticulata*
- Jivaka – *Malaxis acuminata*
- Rishabhaka – *Manilkara hexandra (Roxb.) Dubard* / *Mimusoops hexandra Roxb.*
- Shali – Rice – *Oryza sativa*
- Kasha – *Saccharum spontaneum*
- Shara – *Serratophyllum submersom*
- Darbha – *Saccharum spontaneum*
- Ikshu – Sugarcane – *Saccharum officinarum*
- Twak – Cinnamon – *Cinnamomum zeylanicum*
- Ela – Cardamom – *Elettaria cardamomum*
- Musta – Nut grass (root) – *Cyperus rotundus*
- Rajani – Turmeric (rhizome) – *Curcuma longa*
- Pippali – Long pepper fruit – *Piper longum*

- Agaru – *Aquilaria agallocha*
- Chandana – Sandalwood – *Santalum album*
- Mandukaparni – Gotu Kola – *Centella asiatica*
- Nagakeshara – *Mesua ferrea*
- Shankhapushpi – *Clitoria ternatea*
- Vacha – *Acorus calamus*
- Plava – *Nyctanthes arbor-tristis*
- Yashti – Licorice – *Glycyrrhiza glabra*
- Vidanga – False black pepper – *Embelia ribes*
- Sitopala – Sugar candy
- Sarpi – Cow ghee
- Taila – Sesame oil – *Sesamum indicum*
- Kshaudra – Honey

Table No. 1 Key ingredients of Brahma Rasayana and their pharmacological actions[38]

Name of the ingredient	Pharmacological actions
Shankhapushpi, Vacha	Excellent memory enhancer and improves speaking capabilities.
Amla	Excellent rejuvenator and anti-oxidant.
Pippali, Ela, Twak	Improve strength of respiratory system.
Shatavari	Good for male and female reproductory systems and for gastric complaints.
Punarnava Ikshu Musta	Cleanses and rejuvenates kidney and bladder
Bala	Improves physical and mental strength.
Vidanga	Fights against toxins and microorganisms, bacteria

PLAN OF THE WORK



METHODOLOGY

Experimental animals

Colony inbred strains of Swiss albino male mice 3-4 weeks old and weighing 18-24 gm were used in the study. The female mice were not considered because their hormonal level changes may influence the cognitive behavior of the animal. Animals were obtained from Animal Experimental Laboratory, Madras Medical College, Chennai-03. The animals were kept under standard conditions at 23-25^{0}C, 12 hour light/dark cycle and given standard pellet diet and water *ad libitum.*

The animals were acclimatized to the laboratory conditions for a week prior to the experimentation. Principles of animal handling were strictly adhered and the handling of animals was made under the supervision of Animal Ethics Committee of this institute. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC), Madras Medical College, Chennai-03 which is certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Approval. No: 10/243/CPCSEA dated 10/08/2015

Pharmacological study:

Swiss albino male mice weighing 18-24 gm were divided into five groups each consisting of six animals. The first group was considered as the control group. Cognitive dysfunction was induced by subcutaneous administration of D-Galactose continuously for 7 weeks to all the animals except the 1st group.

Chronic systemic exposure of mice and rats to D-Galactose causes the acceleration of senescence and has been used as an aging model. Chronic D-Galactose exposure induces a spatial memory deficit, neurodegeneration by enhancing caspace mediated apoptosis. It inhibits neurogenesis and neuron migration, as well as increasing oxidative damage and consequent damage of hippocampal neurons by triggering apoptosis cascades leading to neuronal loss and cognitive dysfunction.[29]

Donepezil (5 mg/kg, p.o) was used as the standard drug for this study. Donepezil is a reversible anti-acetylcholinesterase which produces measurable improvement in several cognitive as well as non-cognitive scores in AD. The benefit is ascribed to elevation of acetylcholine level in the cortex, especially in the surviving neurons that project from basal forebrain to cerebral cortex and hippocampus.[40]

The animals were subjected to treatment with vehicle, Brahma Rasayana (test drug) at the dose levels of 1g/kg, 2g/kg p.o and Donepezil (standard drug) 5mg/kg p.o for 30 days after induction of cognitive impairment. The treatment schedule is given in **Table No 2**

Table No.2 Group of animals and their treatment schedule

S.No	**Group**	**Number of animals**	**Treatment (Day 50-79)**
1	Group 1 (control)	6	0.1% w/v of carboxy methyl cellulose was given p.o for 30days.
2	Group 2 (Disease control)	*6*	After 7 weeks, 0.1% w/v of carboxy methyl cellulose was given p.o for 30 days.
3	Group 3 (Donepezil)	*6*	After 7 weeks, **Donepezil** 5mg/kg p.o, was administered for 30 days.
4	Group 4 (Brahma Rasayana, low dose)	6	After 7 weeks, **Brahma Rasayana** 1g/kg p.o, was administered for 30 days.
5	Group 5 (Brahma Rasayana, high dose)	6	After 7 weeks, **Brahma Rasayana** 2g/kg p.o, was administered for 30 days.

The cognitive impairment in all the groups of animals except Group 1 (control) was induced by the administration of D-Galactose (100mg/kg, s.c) daily for 7 weeks continuously. (i.e from day 1 to day 49).

IN VIVO SCREENING

Memory and the locomotor activity were evaluated on 0 day, 49th and 79th day of the study. For assessing memory, Morris water maze and Y-maze test were used. The locomotor activity was studied using Open field test and Actophotometer.

METHODS TO EVALUATE MEMORY

Morris Water Maze Test[41,42]

The Morris water maze test is performed to evaluate spatial working and reference memory. The maze consists of a large circular pool of water with a hidden platform. The platform offers no local cues to guide escape behavior. The animals when allowed in the pool, can escape from swimming by climbing onto the platform and with time the animal apparently learns the spatial location of the platform from any starting position at the circumference of the pool.

Morris water maze consists of a large circular tank (60cm in diameter and 30 cm in height) made of opaque polyvinyl chloride. The pool is filled up to a height of 21 cm with water maintained at around 25^{0}C and rendered opaque by addition of a nontoxic white color (Titanium dioxide powder). The tank was hypothetically divided into four equal quadrants and a platform of 19 cm height was placed in the centre of one of these four quadrants. The platform remains fixed in the position during the training session.

Each animal was subjected to four consecutive trials for four days during which they were allowed to escape on to the hidden platform and allowed to remain there for 20 sec. Escape latency time, i.e the time taken to locate the hidden platform in water maze was noted as an index of acquisition or learning. In case the animal was

unable to locate the hidden platform within 120 sec, it was gently guided by hand to the platform and allowed to remain there for 20 sec. The Morris water maze test was performed on 0, 49th and 79th day of the experiment for all the five groups of animals.

Y-maze test [43,44]

The continuous spontaneous alternation behavior was evaluated by using Y-maze apparatus. The Y-maze apparatus was made of black plastic with three arms (40cm×15cm×35cm) extending from a central platform at 120^0. Each mouse was placed at the end of one arm and allowed to move freely through the maze during a session lasting 5 minutes. Arm entry was defined as the entry of four paws into one arm. The sequence of arm entries were recorded visually. Alternation was defined as multiple entries into three arms (A, B or C) on overlapping triplet sets. The percentage of spontaneous alternation was calculated as the ratio of the actual-to-possible alternations (defined as the total number of arm entries minus 2), multiplied by 100, as shown in the following equation

$$Alternation\ (\%) = \left[\frac{number\ of\ alternations}{total\ arm\ entries - 2}\right] \times 100$$

The Y-maze test were performed on 0, 49th and 79th day of the experiment for all the 5 groups of animals.

METHODS TO EVALUATE LOCOMOTOR ACTIVITY

Open Field Test[45]

The open field apparatus was used for the study of locomotor activity. Open field apparatus consist of a box 95 cm × 95 cm and a height of 16 cm. The floor was

divided into sixteen equal squares by lines drawn on it. A central square was drawn on the middle of the open filed. The mice were centrally placed in the open field apparatus and were allowed to move without restraint inside the area for 5 minutes and ambulation (number of squares crossed by the animal) was noted.

The animals were exposed for two consecutive days to the apparatus for habituation. The open field was cleaned with 5 % water-alcohol solution before behavioral testing to eradicate possible bias due to smells left by previous mice. The open field test was performed on 0, 49th and 79th day of the experiment in all the 5 groups of animals.

Actophotometer[46]

The locomotor activity was measured by using an Actophotometer. The actophotometer consists of a square arena (30 × 30 × 25 cm) with wire mesh bottom, in which the animal moves. Six lights and six photocells are placed in the outer periphery of the bottom in such a way that a single mouse can block only one beam. The movement of the animal interrupts a beam of light falling on a photocell, at which a count is recorded and displayed digitally. The locomotor activity was measured for a period of 5 min. The actophotometer test was performed on 0, 49th and 79th day of the experiment in all the 5 groups of animals.

***EX VIVO* STUDIES**

At the end of study on 79th day, all the animals were sacrificed by cervical dislocation followed by decapitation. The whole brain was surgically removed and transferred to ice cold saline solution and preserved. This was later subjected to neurotransmitter estimation.

ESTIMATION OF NEUROTRANSMITTERS

Neurotransmitters are endogenous chemicals that transmit signals from a neuron to a target cell across a synapse. Glutamate is the major excitatory neurotransmitter in the brain and spinal cord. Excessive glutamate release can lead to excitotoxicity causing cell death. Excitotoxicity has been implicated in ceretain chronic diseases including ischemic stroke, epilepsy, amyotrophic lateral sclerosis, AD, Huntington disease and PD. GABA is released at a majority of fast inhibitory synapses in virtually every part of the brain. Many sedative/tranquilizing drugs act by enhancing the effects of GABA. Acetylcholine is a neurotransmitter at the central and peripheral nervous system. Acetylcholine is involved in memory and attention. Dopamine has a number of important functions in the brain. It plays a critical role in the reward system but dysfunction of the dopamine system is also implicated in Parkinson's disease and schizophrenia. Serotonin is a monoamine neurotransmitter to regulate appetite, sleep, memory and learning, temperature, mood, behavior, muscle contraction, heart and hormone level. It is speculated to have a role in depression. [47,48]

The estimation of Acetylcholinesterase and other neurotransmitter levels was carried out by using the isolated whole brain of the mice. The estimation was carried out for the following:

1) Acetylcholinesterase enzyme
2) Neurotransmitters
    - Glutamate
    - Serotonin
    - Dopamine
    - Adrenaline
    - Nor-adrenaline

Estimation of Acetylcholinesterase (AChE) by Ellman's method [21,49,50]

Acetylcholinesterase (AChE) is an enzyme which breaks down acetylcholine and terminates the neurotransmission process. The most common assay is based on Ellman's method using an alternative substrate acetylthiocholine and 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction results in production of 5-thio-2-nitrobenzoate that has yellow color due to the shift of electrons to the sulfur atom.

Procedure:

A portion of the brain tissue was weighed and homogenized in 0.1M Phosphate buffer (pH 8). 0.4ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1M, pH 8) and 100µl of DTNB. The contents of the cuvette were mixed thoroughly and absorbance was measured at 412 nm in a spectrophotometer. When the absorbance reached a stable value, it was recorded as the basal reading. 20µl of substrate i.e., acetylthiocholine was added and change in absorbance was recorded. Change in the absorbance per minute was determined. Blank: 0.4ml aliquot of the homogenate was added to a cuvette containing 2.7 ml phosphate buffer (0.1M, pH 8) and absorbance was measured at 412nm.

Calculations

The enzyme activity was calculated using the following formula

$$\text{Acetylcholinestease activity (M/ml)} = \frac{A/\text{min} \times Vt}{\varepsilon \times b \times Vs}$$

where,

A/min= Change in absorbance per min

$\varepsilon = 1.361 \times 10^4$ M-1cm-1

b= path length (1 cm)

Vt= Total volume (3.1 ml)

Vs= sample volume (0.4 ml)

The final reading of enzyme activity is expressed as μ moles/minute/mg tissue.

$$\mu \text{ moles/minute/mg of protein} = \frac{\mu \text{ moles /ml sample}}{\text{mg of protein /ml sample dilution}}$$

Estimation of Glutamate[51,52]

A portion of the brain tissue was weighed and homogenized in 0.1M Phosphate buffer (pH 8). 1ml of the supernatant from brain homogenate was evaporated to dryness at 70°C in an oven and the residue is reconstituted in 100 ml of distilled water. Standard solution of glutamate (2.942 mg of glutamate in 10 ml distilled water) and tissue homogenate solutions were spotted on Whatman No. 1 chromatography paper using a micropipette. It was placed on a chamber containing butanol: acetic acid: water (12: 3: 5 v/v) as solvent. When the solvent front reached the top of the paper, it was removed and dried. A second run was performed similarly, after which the papers are dried sprayed with Ninhydrin reagent and placed in an oven at 100°C for 4 minutes. The portions which carry glutamate corresponding with the standard are cut and eluted with 0.005% $CuSO_4$ in 75% ethanol. Their absorbance is read against blank at 515 nm in spectrophotometer.

Calculation

The level of glutamate was calculated by using the following formula:

$$A = \frac{\text{Absorbance of test} \times \text{Standard in mg} \times 1000}{\text{Absorbance standard} \times \text{Volume spotted (10μl)} \times W}$$

where,

A = Amino acid content in μmoles/gram weight tissue

1000 = Conversion factor for gram wet weight tissue

W = weight of the tissue in gram

Estimation of Serotonin, Dopamine, Adrenaline, Nor-adrenaline[52-55]

There are two steps in the estimation of neurotransmitters (Serotonin, Dopamine, Adrenaline, Nor-adrenaline). The first step of sample preparation is common for all the four neurotransmitters and second step is specific to each neurotransmitter.

Preparation of sample solution

A portion of the brain tissue was weighed and homogenized in 5 ml HCl–butanol for about 1 min. The sample was then centrifuged for 10 min at 2000 rpm. An aliquot supernatant phase (1 ml) was taken and added to centrifuge tube containing 2.5 ml heptane and 0.31ml of 0.1 M. HCl. After 10 min of vigorous shaking, the tube was centrifuged under the same conditions as above in order to separate the two phases and the overlaying organic phase was discarded. The aqueous phase was used for the next step.

Estimation of Serotonin

To 0.2 ml aqueous phase (obtained from earlier step), 0.25 ml of OPT (O-pthalaldehyde) reagent was added. The fluorophore was developed by heating to 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, readings were taken at 360-470 nm in the spectrofluorimeter. For serotonin tissue blank, 0.25 ml cont. HCl without OPT was added. Internal Standard: 500 µg/ml of serotonin was prepared in distilled water: HCl-butanol in 1:2 ratio.

Estimation of Dopamine

To 0.2 ml aqueous phase (obtained from earlier step), 0.05 ml of 0.4 M HCl and 0.1 ml of EDTA / Sodium acetate buffer (PH 6.9) were added, followed by 0.1 ml iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.1 ml Na_2SO_3 solution. 0.1 ml Acetic acid was added after 1.5 min.

The solution was then heated to 100°C for 6 min. When the sample reached room temperature, excitation and emission spectra were read from the Spectrofluorimeter. The readings were taken at 330-375 nm. Tissue blanks for Dopamine was prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine). Internal Standard: 500 µg/ml each of dopamine was prepared in distilled water: HCl-butanol in 1:2 ratio.

Estimation of Adrenaline and Nor-adrenaline

To 0.2 ml aqueous phase (obtained from earlier step), 0.05 ml of 0.4 M HCl and 0.1 ml of EDTA / Sodium acetate buffer (PH 6.9) were added, followed by 0.1 ml iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.1 ml Na_2SO_3 solution. 0.1 ml Acetic acid was added after 1.5 min. The solution was then heated to 100°C for 6 min. When the sample reached room temperature, excitation and emission spectra were read from the Spectrofluorimeter. The readings were taken at 395-485 nm. Tissue blanks for adrenaline and nor-adrenaline were prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine). Internal Standard: 500 µg/ml each of adrenaline/ nor-adrenaline was prepared in distilled water: HCl-butanol in 1:2 ratio.

Statistical analysis

The statistical analysis was performed using Graph Pad Prism software version 5.0. All the results were expressed as mean ± SEM. The data were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's Comparison test. The p values <0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

IN VIVO SCREENING

EVALUATION OF SPATIAL MEMORY

Morris water maze test

Morris water maze test is more specific for spatial memory. This test is a widely accepted experimental model for the assessment of cognitive skills. Typically, cognition impaired induced animals exhibit an increase in time for escape latency indicating a loss of visual cues to escape onto the platform. The escape latencies of all the animals tested are given in **Table 3**.

Table 3: Effect of BR on Escape latencies of mice in Morris water maze

S.No	Groups	Escape latency in seconds		
		0 day	49th day	79th day
1	Control	20±3.73	19.17±2.98	19.33±3.13
2	Disease control (D-Galactose, 100mg/kg)	21.00±2.37	69.33±3.21***	79.00±8.01***
3	Standard (Donepezil, 5 mg/kg)	21.17±2.04	69.50±6.96***	22.00±3.88
4	BR Low dose (1g/kg)	20.33±3.94	68.67±8.33***	46.17±3.96**
5	BR High dose (2g/kg)	20.50±2.93	68.50±2.54***	25.17±3.498

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001.
One way ANOVA followed by Dunnet's test.

From the table no. 3 it can be seen that on day 0, there is no significant difference in the escape latency time between the control animals and animals in groups II, III, IV and V.

On day 49, there was a significant ($p < 0.001$) increase in the escape latency produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg, p.o) and standard drug Donepezil 5 mg/kg p.o also showed significant ($p<0.001$, $p < 0.001$ and $p<0.001$ for Group III, IV and Group V respectively) increase in the escape latency when compared with Group I (control) animals. It clearly indicates that D-Galactose induced spatial memory deficits in all the animals except the control group.

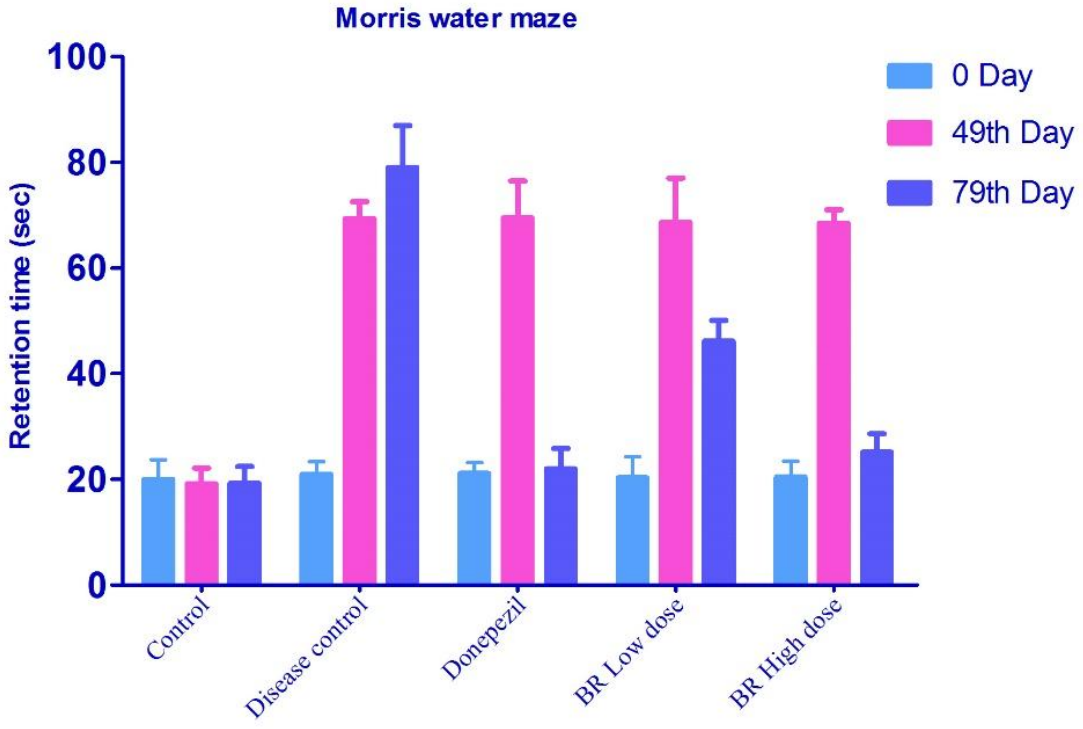


Fig 1: Effects of BR on escape latencies of mice in Morris water maze test. Values are expressed in Mean ± S.E.M, n=6 One way ANOVA followed by Dunnet's test.

On day 79, there was a significant ($p< 0.001$) increase in the escape latency produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. There was a significant decrease in escape latency time in standard Donepezil treated group of animals. This indicates that Donepezil (5 mg/kg) has shown an improvement in cognition. Donepezil is a known anticholinesterase drug which is known to inhibit the actions of AChE. It improves Ach levels in the brain thus leading to improvement in cognition.

There was a improvement in the escape latency time but still it was higher than the 0 day reading in the BR low dose treated group (1g/kg). In the animals treated with the high dose (BR 2g/kg), there was a marked decrease in escape latency time. The escape latency time came back near to the 0 day reading. This clearly indicates that BR at the dose of 2g/kg has a significant cognition enhancing potential which is comparable with that of the standard drug Donepezil at the dose of 5 mg/kg.

Y-Maze test

Y-maze test is one of the simplest versions of spontaneous alternation test which is used to measure memory. The ability to alternate requires the mice to know which arm they have already visited. Normal mice are expected to exhibit an alternation percentage of 60-70. The animals which were injected with D-Galactose had a reduced spontaneous alternation but animals treated with BR produced a significant increase in alternation which was comparable to the untreated control which in turn indicates the increased spatial working memory of the animals.

Table 4: Effect of BR on spontaneous alternation of mice in Y-maze

S.No	Groups	Percentage spontaneous alternation		
		0 day	**49th day**	**79th day**
1	Control	75.25±0.29	76.46±0.57	76.49±0.34
2	Disease control (D-Galactose, 100mg/kg)	76.12±0.15	11.65±0.24***	10.39±0.25***
3	Standard (Donepezil, 5 mg/kg)	75.74±0.50	11.67±0.25***	66.03±0.20***
4	BR Low dose (1g/kg)	75.65±0.25	12.11±0.42***	51.28±0.31***
5	BR High dose (2g/kg)	75.73±0.39	11.83±0.51***	63.26±0.84***

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001.
One way ANOVA followed by Dunnet's test.

From the **Table 4**, it can be seen that on day 0, there is no significant difference in the percentage spontaneous alternation between the control animals and animals in groups II, III, IV and V.

On day 49, there was a significant ($p < 0.001$) decrease in the percentage spontaneous alternation produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg, p.o) and standard drug Donepezil 5 mg/kg p.o also showed significant ($p<0.001$, $p< 0.001$ and $p<0.001$ for Group III, IV and Group V respectively) decrease in the percentage spontaneous alternation when compared with Group I (control) animals. It clearly indicates that D-Galactose induced spatial memory deficits in all the animals except control group.

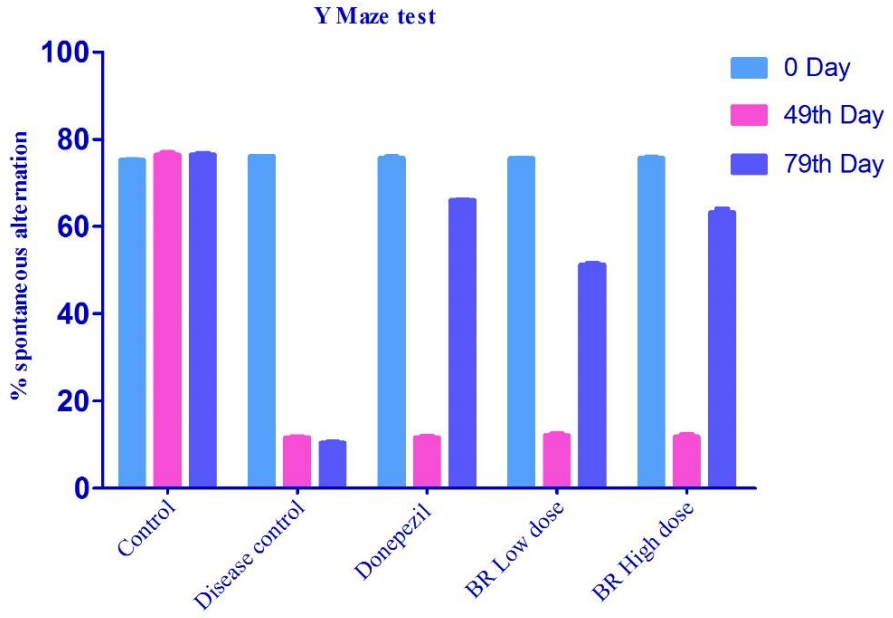


Fig 2: Effects of BR on spontaneous alternation of mice in y-maze test. Values are expressed in Mean ± S.E.M, n=6 One way ANOVA followed by Dunnet's test.

On day 79, there was a significant ($p < 0.001$) decrease in the percentage spontaneous alternation produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. There was a significant increase in the percentage spontaneous alternation in standard Donepezil treated group of animals. This indicates that Donepezil (5 mg/kg) has shown an improvement in cognition.

There was an improvement in the percentage spontaneous alternation but still it was lower than the 0 day reading in the BR low dose treated group (1g/kg). In the animals treated with the high dose (BR 2g/kg), there was a marked increase in percentage spontaneous alternation. This clearly indicates that BR at the dose of 2g/kg has a significant cognition enhancing potential which is comparable with that of the standard drug Donepezil at the dose of 5 mg/kg. p.o

EVALUATION OF LOCOMOTOR ACTIVITY

Open field test

The locomotor activity of the animal was measured by using open field apparatus. In this test, the lines crossed by the animals were noted and compared with control animals. The units are tabulated in Table 5

Table 5: Effect of BR on number of line crossings of mice in open field test

S.No	Groups	Number of line crossings		
		0 day	**49th day**	**79th day**
1	Control	90.17±3.63	75.67±2.75	94.33±2.30
2	Disease control (D-Galactose, 100mg/kg)	86.50±3.99	37.50±3.03***	35.00±4.88***
3	Standard (Donepezil, 5mg/kg)	89.00±4.95	38.67±3.11***	88.00±4.40
4	BR Low dose (1g/kg	88.50±4.27	38.33±3.21***	84.00±4.27
5	BR High dose (2g/kg)	88.17±3.61	38.17±2.65***	85.50±4.39

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnet's test.

From the table 5, it can be seen that on day 0, there is no significant difference in the number of line crossings between the control animals and animals in groups II, III, IV and V.

On day 49, there was a significant (p< 0.001) decrease in the number of line crossings produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg, p.o) and standard drug Donepezil 5 mg/kg p.o also showed significant (p<0.001, p< 0.001 and p<0.001

for Group III, IV and Group V respectively) decrease in the number of line crossings when compared with Group I (control) animals. Cognition is known to induce decreased locomotor activity. A decrease in locomotor activity in all the groups which were administered D-Galactose indicates development of cognitive impairment.

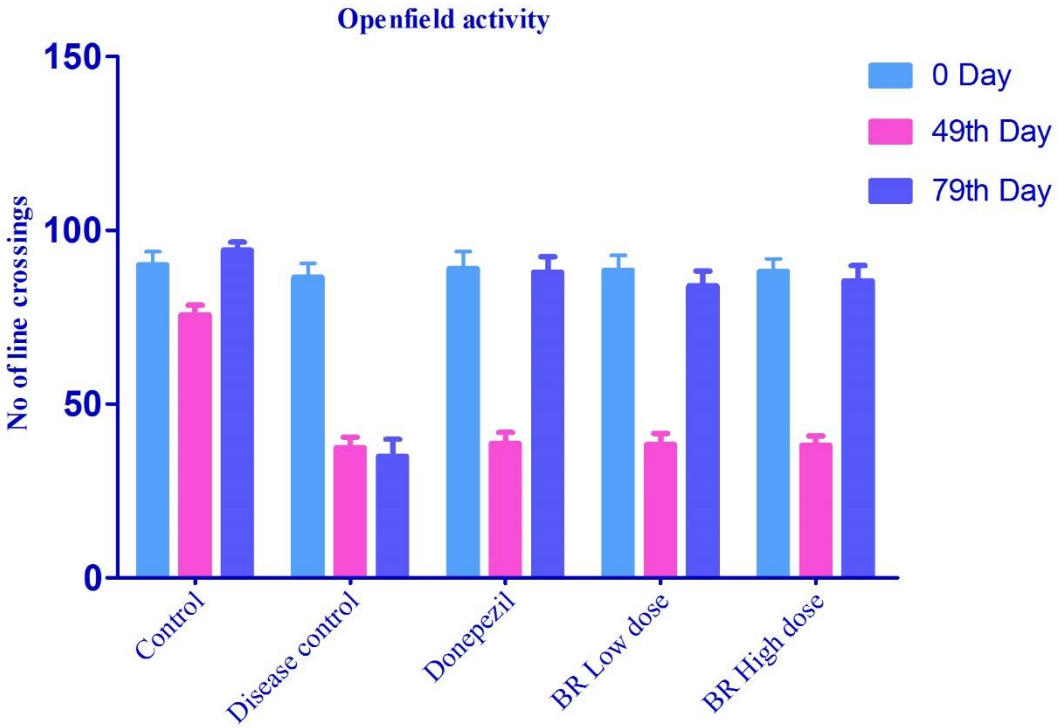


Fig 3: Effects of BR on number of line crossings of mice in open field test. Values are expressed in Mean ± S.E.M, n=6 One way ANOVA followed by Dunnet's test.

On day 79, there was a significant ($p< 0.001$) decrease in the number of line crossings produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. There was a significant increase in the number of line

crossings in standard Donepezil treated group of animals. This indicates that Donepezil (5 mg/kg) has shown an improvement in cognition.

There was a good improvement in the number of line crossings and it was near the 0 day reading in the BR low dose treated group (1g/kg). In the animals treated with the high dose (BR 2g/kg), there was a marked increase in number of line crossings as compared to that of the standard treated group. This clearly indicates that BR at the dose of 2g/kg has a significant cognition enhancing potential which is comparable with that of the standard drug Donepezil at the dose of 5 mg/kg p.o

Actophotometer

The locomotor activity of the animals were evaluated by using actophotometer. The readings are tabulated in table 6.

Table 6: **Effect of BR on evaluation of locomotor activity using Actophotometer**

S.No	Groups	Activity scores		
		0 day	**49th day**	**79th day**
1	Control	127.5±5.33	117.2±4.03	125.7±8.30
2	Disease control (D-Galactose, 100mg/kg)	121.2±6.23	52.33±2.51***	51.83±3.27***
3	Standard (Donepezil, 5mg/kg)	124.2±8.34	51.67±2.25***	97.50±5.52*
4	BR Low dose (1g/kg)	128.2±4.22	51.33±2.91***	78.33±8.81***
5	BR High dose (2g/kg)	126.0±6.27	51.83±2.59***	95.17±3.13*

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001.
One way ANOVA followed by Dunnet's test.

From the table 6, it can be seen that on day 0, there is no significant difference in the activity scores between the control animals and animals in groups II, III, IV and V.

On day 49, there was a significant ($p < 0.001$) decrease in the activity scores produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg, p.o) and standard drug Donepezil 5 mg/kg p.o also showed significant ($p<0.001$, $p < 0.001$ and $p<0.001$ for Group III, IV and Group V respectively) decrease in the number of line crossings when compared with Group I (control) animals.

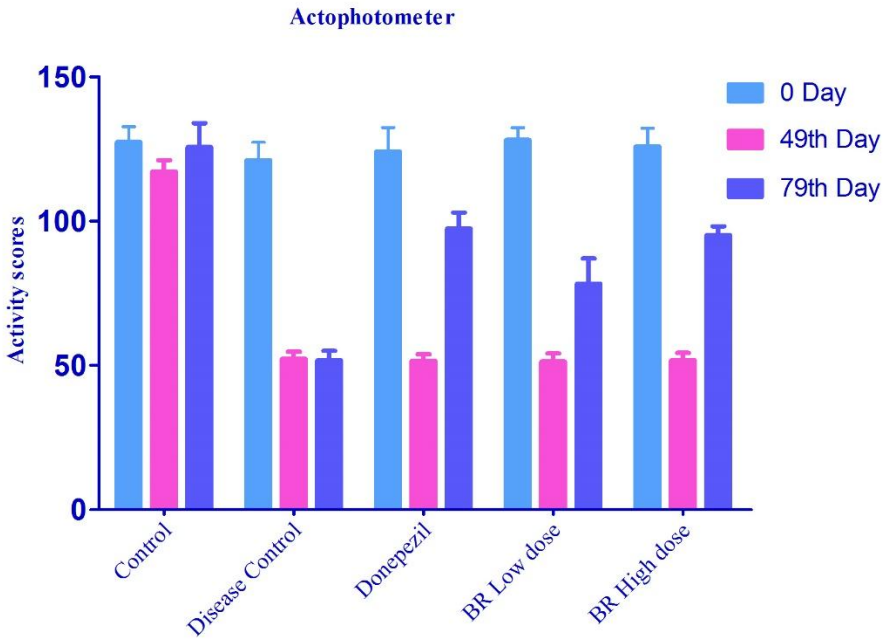


Fig 4: Effects of BR On activity scores of mice in actophotometer. Values are expressed in Mean ± S.E.M, n=6 One way ANOVA followed by Dunnet's test.

On day 79, there was a significant ($p < 0.001$) decrease in the activity scores produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. There was a significant increase in the activity scores in standard

Donepezil ($p<0.05$) treated group of animals. This indicates that Donepezil (5 mg/kg), a known cognitive enhancer has shown an improvement in cognition.

There was an improvement in the activity scores in the BR low dose ($p<0.001$) treated group (1g/kg). In the animals treated with the high dose (BR 2g/kg), there was a good increase in activity scores compared to that of the standard treated group. This clearly indicates that BR at the dose of 2g/kg has a significant cognition enhancing potential which is comparable with that of the standard drug Donepezil at the dose of 5 mg/kg. p.o

From the above *in vivo* studies it can be seen that BR at the higher dose of 2 g/kg is able to improve memory as seen in the Morris water maze and Y-maze studies. There is also an improvement in the locomotor activity as seen in the Open field test and Actophotometer studies. From this it can be concluded that BR at the dose of 2g/kg is a cognition enhancer against D-Galactose induced cognition impairment.

EX VIVO SCREENING

Acetylcholinesterase activity

Both nicotinic and muscarinic cholinergic receptors are involved in cognitive and memory functions and several studies have suggested their roles in dementia. Marked cholinergic deficit is a hallmark of the pathogenesis of AD and various drugs including AChE inhibitors have been designed to target this deficit. Initially, cholinergic deficit was thought to be a muscarinic nature, but recent studies show a specific loss of nicotinic acetyl choline receptors and marked loss of cholinergic neurons.

Table 7: Effect of BR on the amount of Acetylcholinesterase enzyme

S.No	Groups	Amount of AChE (µmoles/min/mg of protein)
1	Control	23.11±0.25
2	Disease control (D-Galactose, 100mg/kg)	32.07±0.13***
3	Standard (Donepezil, 5mg/kg)	24.35±0.23***
4	BR Low dose (1g/kg)	30.83±0.12***
5	BR High dose (2g/kg)	25.95±0.16***

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnet's test.

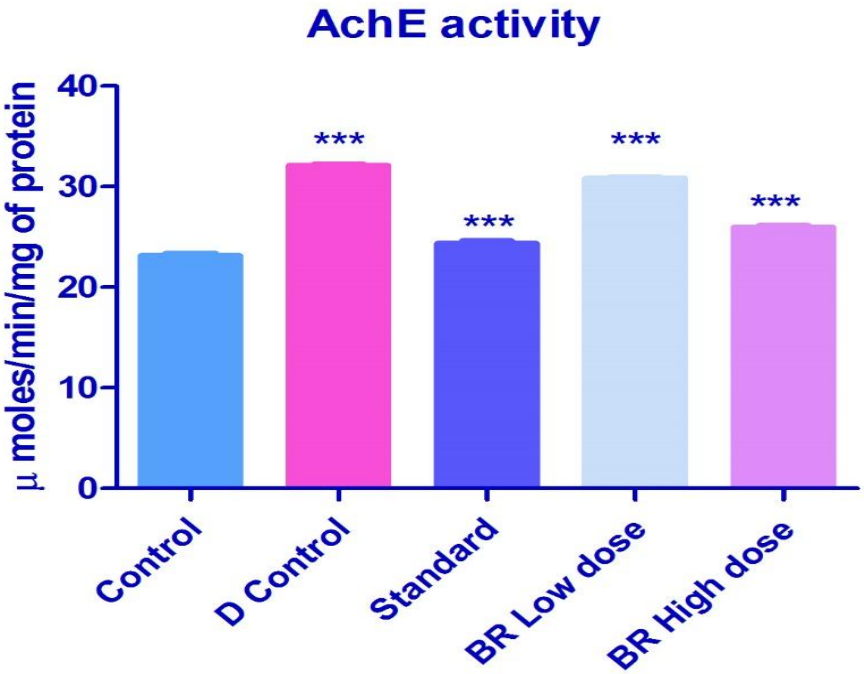


Fig 5: Effects of BR on the amount of acetylcholinesterase enzyme in mice. The enzyme activity is expressed in µmoles/min/mg of protein. Values are expressed in Mean ± S.E.M, n=6 One way ANOVA followed by Dunnet's test.

From the **Table 7**, it is seen that group which is injected with D-Galactose there was an increase AChE levels. The level has been significantly reduced in the Donepezil (5 mg/kg) treated group. This is expected since Donepezil is a known Anticholinesterase agent.

In the BR treated group, at the lower dose of 1g/kg the AChE levels showed a reduction from the disease group. But in the animals treated with higher dose of BR there was a significant reduction in the AChE levels. However this reduction is not on par with the standard drug Donepezil.

From these results it can be postulated that BR is able to inhibit AChE levels, thus increasing the levels of Ach in the brain.

Glutamate

The levels of Glutamate in the various groups of animals is given in Table 8.

Table 8: **Effect of BR on the level of Glutamate**

S.No	Groups	Amount of Glutamate present in µg/mg weight of tissue
1	Control	3.5430 ± 0.16
2	Disease control (D-Galactose, 100mg/kg)	6.3150 ± 0.04***
3	Standard (Donepezil, 5mg/kg)	4.3950 ± 0.03***
4	BR Low dose (1g/kg)	5.3540 ± 0.04***
5	BR High dose (2g/kg)	5.1230 ± 0.06***

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001.
One way ANOVA followed by Dunnet's test

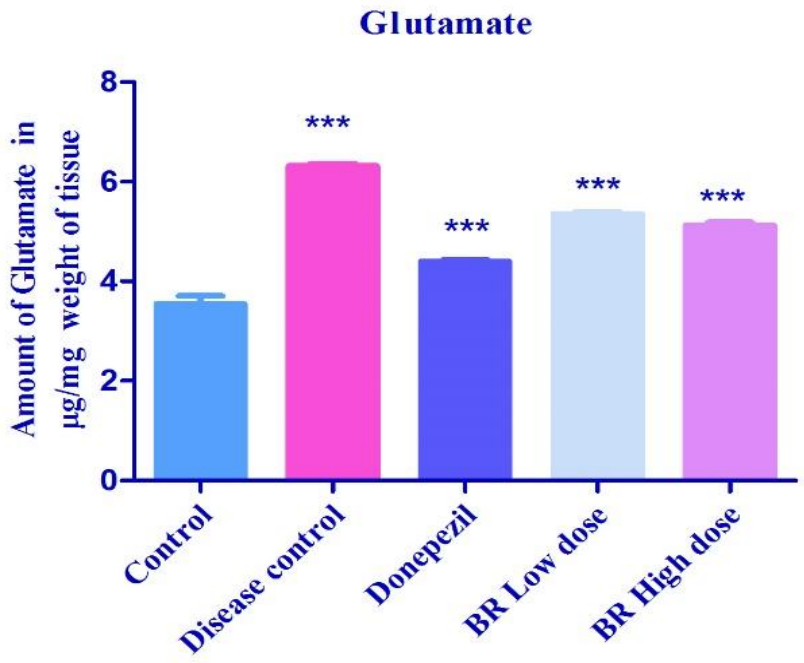


Fig 6: Effects of BR On the amount of glutamate in mice. It is expressed in µg/mg wt of tissue. Values are expressed in Mean ± S.E.M, n=6 . One way ANOVA followed by Dunnet's test.

There was a significant ($p < 0.001$) increase in the Glutamte levels (µg/mg wt of tissue) produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg p.o) and standard drug Donepezil (5 mg/kg p.o) showed a significant ($p<0.001$, $p< 0.001$ and $p<0.001$ for Group III, IV and Group V respectively) decrease in the elevated amount of Glutamate when compared with Group II (D-Galactose injected) animals.

Serotonin, Dopamine, Adrenaline and Nor-Adrenaline

The amount of above mentioned neurotransmitters were estimated in the isolated brain tissue. The observations are given in **Table 9** and **Fig 7**

Table 9: **Effect of BR on the various neurotransmitters**

S.No	Groups	Amount of neurotransmitters present in µg/mg weight of tissue			
		Serotonin	**Dopamine**	**Adrenaline**	**Noradrenaline**
1	Control	0.5433±0.01	0.2135±0.004	31.99±0.44	114.5±0.89
2	Disease control (D-Galactose, 100mg/kg)	0.0698±0.002***	0.0407±0.0003***	17.74±0.51***	65.22±0.83***
3	Standard (Donepezil, 5mg/kg)	0.3350±0.006***	0.1435±0.002***	29.48±0.37***	96.92±0.64***
4	BR Low dose (1g/kg)	0.2232±0.015***	0.0826±0.003***	21.50±0.49***	82.56±0.61***
5	BR High dose (2g/kg)	0.2763±0.007***	0.0895±0.002***	27.37±0.39***	85.91±1.02***

Values are expressed in Mean ± S.E.M, n=6 *p<0.05, **p<0.01, ***p<0.001.
One way ANOVA followed by Dunnet's test

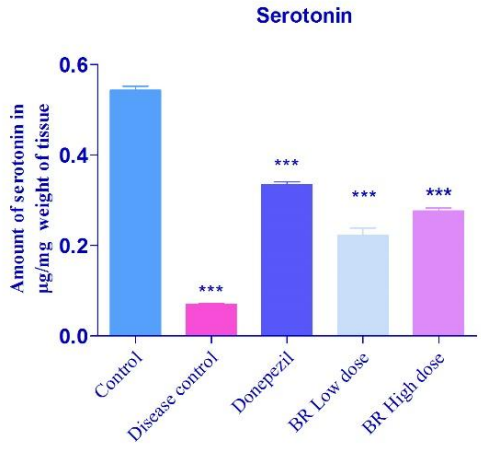


Fig 7a

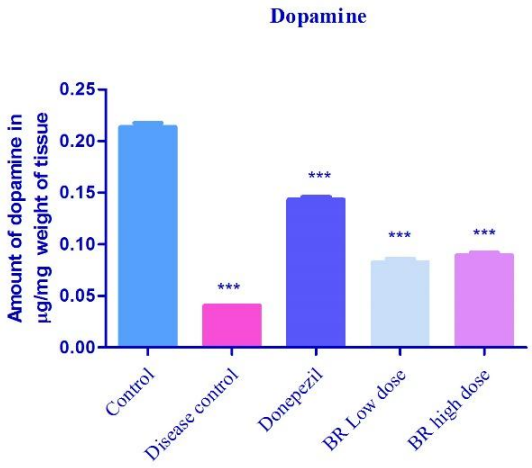


Fig 7b

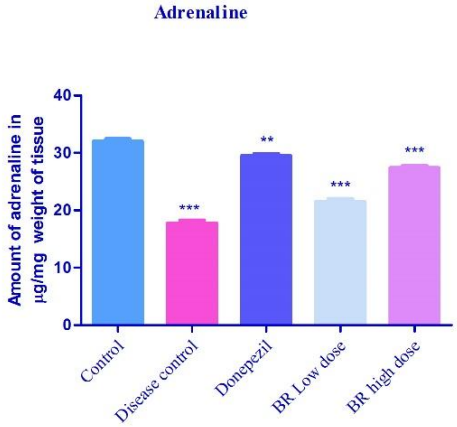


Fig 7c

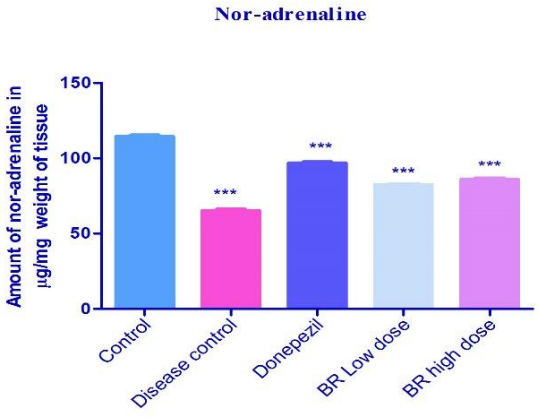


Fig 7d

Fig 7a,7b,7c,7d : Effects of BR On the levels of serotonin, dopamine, adrenaline and nor-adrenaline in mice respectively. It is expressed in µg/mg wt of tissue. Values are expressed in Mean ± S.E.M, n=6 .One way ANOVA followed by Dunnet's test.

There was a significant ($p< 0.001$) decrease in the amount of serotonin, dopamine, adrenaline and nor-adrenaline (µg/mg wt of tissue) produced by Group II (D-Galactose injected) animals when compared with Group I (control) animals. Treatment with BR (1g/kg and 2g/kg p.o) and standard drug Donepezil (5mg/kg p.o) showed a significant ($p<0.001$, $p< 0.001$ and $p<0.001$ for Group III, IV and Group V respectively) improvement in the levels of serotonin, dopamine, adrenaline and nor-adrenaline when compared with group II animals. From the data obtained in table 8 it can be concluded that the levels of Serotonin, Dopamine, Adrenaline and Nor-adrenaline were reduced with the D-Galactose injected group. Donepezil and higher dose of BR (2g/kg) treated group of animals, showed a significant increase in neurotransmitter levels as compared to group II animals. The lower dose of BR (1g/kg) also showed a significant increase in neurotransmitter levels, but it is lesser than that of BR higher dose treated animals.

SUMMARY

Cognitive impairment is a classic sign of various conditions such as Alzheimer's disease (AD), Pick's disease, Parkinson's disease, Lewy body disease, Huntington's disease, progressive supranuclear palsy and cerebellar degeneration. In market there are a few drugs available for the improvement of cognition. There are several Ayurvedic formulations which are being marketed for this condition. Brahma Rasayana is one such formulation with the memory boosting capacity.

This study was undertaken to scientifically validate the claim of BR as a cognitive enhancer. The model used for the study was the D-Galactose which is one of the commonly used models for cognition impairment.

Donepezil was used as a standard drug for comparison. The models used to test cognition were Morris water maze and Y-maze test for evaluating spatial memory, Actophotometer and open field tests were used for evaluation of locomotor activity. On all these studies it was seen that BR, especially at the higher dose (i.e 2g/kg p.o), was able to reverse the cognitive impairment induced by D-Galactose. This activity was almost the same as that of Donepezil.

In order to establish the mechanism of action, the brain levels of acetylcholinesterase (Ach being one of the important neurotransmitters in cognition) and neurotransmitters like Serotonin, Dopamine, Adrenaline, Nor-adrenaline and Glutamate were estimated. Brahma Rasayana at the higher dose (i.e 2g/kg p.o), showed a good anti-cholinesterase activity. The levels of other neurotransmitters like Serotonin, Dopamine, Adrenaline, Nor-adrenaline were increased markedly as compared to the disease group animals. The level of Glutamate was decreased in Donepezil and BR treated group as compared to disease group of animals, which indicates that the excitotoxicity of glutamate was reduced by the both standard and BR.

CONCLUSION

From the study it is concluded that Brahma Rasayana, which is marketed with claims for use in the treatment of drowsiness, fatigue, mental weakness, senility/ progeriasis, aging and disturbed memory, has cognition enhancing property at a higher dose of 2g/kg and this action is mediated mainly by the Anticholinesterase activity. It has also shown an improvement in the levels of neurotransmitters like Serotonin, Dopamine, Adrenaline, Nor-adrenaline and Glutamate, which are altered in cognition impairment induced by D-Galactose. This study validates the Ayurvedic claim of Brahma Rasayana as a memory enhancer.

This claim can be further strengthened by carrying out a systematic clinical study using Brahma Rasayana in cognition impaired patients.

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CERTIFICATE

This is to certify that Mr. **K. SATHIYASEELAN,** M.Pharm II year, Institute of Pharmacology, Madras Medical College, Chennai – 600003 had submitted his protocol (Part B Application) 10/243/CPCSEA for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai – 600003.

TITLE: SCIENTIFIC EVALUATION OF BRAHMA RASAYANA AS COGNITIVE ENHANCER IN D-GALACTOSE INDUCED COGNITIVE IMPAIRMENT IN MICE.

The Animal Ethical Clearance Committee experts screened his proposal No: 10/243/CPCSEA and have given clearance in the meeting held on 10/08/2015 at Dean's Chamber in Madras Medical College, Chennai – 600003. His study involves only Swiss Albino mice.

Signature

9/3/2016

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