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ORIGINAL ARTICLE

Elucidation of Molecular Mechanism(s) of Cognition Enhancing Activity of Bacomind®: A Standardized Extract of Bacopa Monnieri

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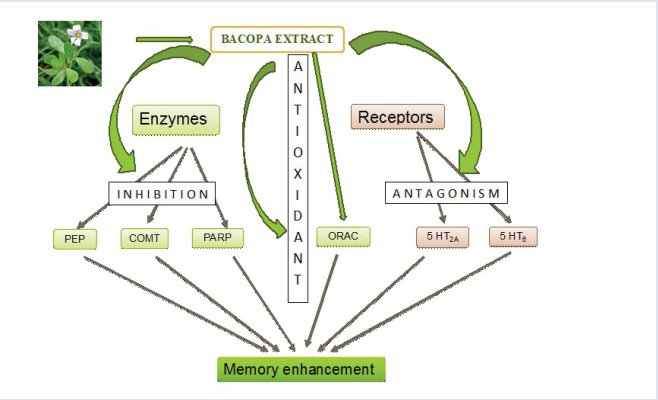
ABSTRACT

Background: *Bacopa monnieri* (L.) Wettst., commonly known as Brahmi, is renowned in Indian traditional system for its potent memory enhancing activity, which has been validated by various scientific studies. **Objective:** The objective of this study was to understand the molecular mechanism of memory enhancing activity of BacoMind® (BM), a standardized extract of *B. monnieri*. **Materials and Methods:** BM was screened *in vitro* in a panel of cell-free and receptor-transfected cell assays. The purified enzymes/membrane homogenates/cells were incubated with substrate/standard ligand in the absence or presence of the test compound. The IC_{50} values and EC_{50} values were determined by nonlinear regression analysis of the concentration–response curves generated with mean replicate values using Hill equation curve fitting. **Results:** BM was found to inhibit three enzymes; Catechol-O-methyl transferase (COMT), Prolyl endopeptidase (PEP), and Poly (ADP-ribose) polymerase (PARP). It also had an antagonistic effect on serotonin 6 and 2A (5-HT_6 and 5-HT_{2A}) receptors known to influence the different neurological pathways, associated with memory and learning disorders, age-associated memory impairment. **Conclusion:** BM was found to inhibit three enzymes namely, Catechol-O-methyl transferase (COMT), Prolyl endopeptidase (PEP), and Poly (ADP-ribose) polymerase (PARP). It also exhibited an antagonistic effect on 5-HT_6 and 5-HT_{2A} receptors.

Key words: Bacopa monnieri, Bacomind, cognition, enzyme inhibition, learning and memory, mechanism of action, serotonin receptors,

SUMMARY

This study was conducted to understand the molecular mechanism of memory enhancing activity of a standardized extract of *B. monnieri* by was screening it *in vitro* in a panel of cell-free and receptor-transfected cell assays. The purified enzymes/membrane homogenates/cells were incubated with substrate/standard ligand in the absence or presence of the test compound. BM was found to inhibit three enzymes; Catechol-O-methyl transferase (COMT), Prolyl endopeptidase (PEP), and Poly (ADP-ribose) polymerase (PARP). It also had an antagonistic effect on serotonin $_6$ and $_{2A}$ (5-HT_6 and 5-HT_{2A}) receptors, known to influence the different neurological pathways, associated with memory and learning disorders, age-associated memory impairment.



Abbreviations used: HTRF: Homogenous time resolved fluorescence, cAMP: Cyclic adenosine monophosphate, CHO: Chinese hamster ovary, RFU: Relative fluorescence unit, pNP: Para nitro phenol, AMC: 7-amino-4-methylcoumarin, ELISA: Enzyme linked immunosorbent assay, Z-Pro-Pro-CHO: Z-prolyl-prolinal, HEK: Human embryonic kidney, TE: Trolox equivalent.

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INTRODUCTION

Bacopa monnieri (L.) Wettst., (family: Plantaginaceae) also called Brahmi, is found throughout India. Many Indian traditional literatures like *Athar-Ved*, *Charak Samhita* and *Susrutu Samhita* have detailed the medicinal importance of *B. monnieri*. The traditional knowledge is aptly supported by modern scientific literature, which emphasizes the medicinal importance of *B. monnieri*. In addition to cognitive properties, *B. monnieri* has been reported to possess anti-ulcerogenic, anti-oxidant, adaptogenic, anti-anxiety, anti-depressant and hepatoprotective activities.[1] It contains alkaloids (nicotine and herpestine), flavonoids (luteolin and apigenin) and saponins (bacoside A3, bacopaside I, bacopaside II, jujubogenin isomer of bacopa saponin C, bacopa saponin C). Various preclinical and clinical studies have reported *B. monnieri* to be effective in improving memory and cognition. It has shown significant reduction in forgetting the acquired information and improvement in memory acquisition and retention in healthy older individuals.[2] The capacity of *B. monnieri* to improve complications related to neurodegenerative

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disorders has also been studied and it was found to reduce the deposition of β-amyloid protein in animal model of Alzheimer's.[3] The potential of *B. monnieri* has also been studied as an anti-parkinsonian agent using *C. elegans* model,[4] which signifies its importance in neurodegenerative disorders. Even though the role of *B. monnieri* as a memory and cognition enhancer has been accepted traditionally and proved in scientific literature with profuse evidences, the ambiguity about its mechanism of action still remains to be resolved. We studied the effect of a methanolic extract of *B. monnieri* standardized to 40% bacosides, BacoMind™ (BM), on the different molecular targets associated with memory and cognition in order to understand its mechanism of memory enhancing action.

Memory is not a unitary function as different memory and learning forms are sub served by different neurological pathways, which are closely interweaved with each other.[5] The major neurological pathways that are involved in the memory and cognition include cholinergic, dopaminergic, serotoninergic pathway, and neuroprotective or antioxidant pathway[6]. Among the serotoninergic pathway targets, down regulation of $5HT_{2a}$, $5HT_3$[7] and $5HT_6$ receptors[8] have been reported to improve memory. The inhibition of enzymes in the cholinergic pathway like acetyl cholinesterase (AChE) and butryl cholinesterase (BuChE), prevents the degradation of cholinergic neurotransmitters and improves the cholinergic transmission in the brain[9] helping to improve long-term memory processes. In the dopaminergic pathway, the inhibition of monoamine oxidase B (MAO-B), which breaks down monoamines like dopamine, has been found to be beneficial for memory and learning.[10] Oxidative stress in the brain can impair memory and learning. Brain is susceptible to oxidative stress as it is a region with high metabolic activity and there are high levels of unsaturated lipids and pro-oxidant iron.[11]

Even though the cognition enhancing properties of BM has been demonstrated in preclinical and clinical studies,[12,13] its mechanism of action has not been elucidated. The objective of our study was to understand the effect of BM on some of the targets that are associated with central nervous system disorders in order to elucidate its mechanism of memory and cognition enhancing activity.

MATERIALS AND METHODS

The effect of BM on various receptor and enzymes was assessed by using different validated *in vitro* assays.

Receptor-based assays

BM was tested in receptor binding assays for Muscarinic 1 (M_1), Serotonin 3 ($5\text{-}HT_3$), Gamma amino butyric acid (GABA), Adrenoreceptor alpha 2A (α_{2A}), N-methyl-D-aspartate (NMDA) and Glycine site (strychnine insensitive) receptors and in agonist/antagonist functional assays for Canabinoid 1 (CB_1) Dopamine 1 (D_1), $5\text{-}HT_{2A}$ and $5\text{-}HT_6$ receptors to determine its interaction with these receptors.

For radio ligand binding assay the cell membrane homogenates were incubated for 90 min at 22°C with labeled standard ligand in the absence or presence BM in a buffer containing 20 mM Tris-HCl (pH 7.4). Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters presoaked with 0.3% polyethyleneimine and rinsed several times with ice-cold 50 mM Tris-HCl. The filters were dried and then radioactivity was counted in a scintillation counter (Topcount, Packard).

BM was tested at a concentration of 5 μg/mL and 25 μg/mL for GABA and $\alpha\text{-}_{2A}$ binding assays. For all other binding assays a concentration of 10 μg/mL and 100 μg/mL was used for testing BM.

For functional assay, respective cells were suspended in DMEM buffer and were distributed in microplates. The fluorescent probe was then added into each well and equilibrated with the cells for 60 min at 37°C then 15 min at 22°C.

Thereafter, the test compound, reference antagonist or HBSS buffer was added and then 5 min later 3 nM of reference agonist or HBSS buffer (basal control), and the changes in fluorescence intensity was measured. The results were expressed as percent inhibition of the control response to 3 nM reference agonist [Table 1].

BM was evaluated at 5 μg/mL and 25 μg/mL in various functional assays to check its agonist/antagonist effect on the selected receptors. The agonist effect was determined by studying the binding of BM to different receptors as compared to control (stimulated with agonist).

For the binding and agonist assays, results were expressed as percent activity of the control value in the presence of BM, calculated using the formula:

$$\text{Percent (\%) of control agonist response} = \frac{\text{Measured response}}{\text{Control response}} X\ 100$$

For antagonistic assays, the results were expressed as % inhibition of control agonist response calculated using the formula:

$$\text{Percent (\%)inhibition of control agonist response} = 100 - \frac{\text{Measured response}}{\text{Control response}} X\ 100$$

The EC_{50} values (concentration producing a half-maximal response) and IC_{50} values (concentration causing a half-maximal inhibition of the control agonist response) were determined by non-linear regression analysis of the concentration–response curves generated with mean replicate values using Hill equation curve fitting.

Enzyme-inhibition assays

The effect of BM on 16 enzymes listed in [Table 2] was evaluated using different assay formats. Purified enzymes were incubated with their respective substrates in presence and absence of BM and the colored/fluorescent product formed was measured using microplate reader [Table 2].

Percentage inhibition was calculated using the formula:

$$Percent\ inhibition = \frac{\text{Absorbance/RFU of control} - \text{Absorbance/RFU of sample}}{\text{Absorbance/RFU of control}} X\ 100$$

IC_{50}, the concentration of inhibitor required to inhibit the activity of enzyme by 50%, was calculated by log-probit analysis, using Graphpad Prism® version 5.01 software. All the assays were conducted in triplicate and the results were expressed as Mean ± S.D.

RESULTS

A 43.3 ± 9.54% inhibition of control specific binding by BM for M_1 receptor was the highest inhibition observed among all the selected receptors at the tested concentration. [Table 1] represents the percent inhibition and percent of control specific binding by BM in different receptor binding assays. Since, the results of binding of BM to the selected receptors were not significant, further studies using functional assays for evaluating the agonist/antagonistic effects for these receptors were not performed.

BM exhibited agonist effect on Canabinoid 1 (CB_1) receptors and the percent binding activity at 25 μg/mL with reference to control for $5\text{-}HT_{2A}$ and CB_1 receptors was found to be 33.3 ± 1.41% and 105.4 ± 0.42%, respectively [Figure 1].

BM displayed considerable potency in displacement of respective agonist ligands from D_1, $5\text{-}HT_{2A}$ and $5\text{-}HT_6$ receptors [Figure 2]. A very insignificant antagonist response was exhibited by BM in $5\text{-}HT_{4e}$ antagonist assay.

The notable antagonist effect exhibited by BM at the $5\text{-}HT_6$ receptors was further confirmed by determining its IC_{50} in $5\text{-}HT_6$ functional assays. BM was tested in $5\text{-}HT_6$ functional assays at concentrations ranging from 4.29 to 50 μg/mL and its IC_{50} in $5\text{-}HT_6$ antagonistic assay was found to be 52 ± 1.2 μg/mL.

Out of the 16 enzyme evaluated, BM showed inhibitory activity on three enzymes. The IC_{50} value of BM in PEP, COMT and PARP inhibition

Table 1: Details of different receptor based assays

Assay	Origin	Detection method	Product measured	Reference compound
5-HT_{4e} Receptor (Agonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	Serotonin
5-HT_{4e} Receptor (Antagonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	GR 113808
5-HT_6 Receptor (Antagonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	Methiothepin
5-HT_6 Receptor (Agonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	Serotonin
5-HT_{2A} Receptor (Antagonist effect)	Human recombinant (HEK-293 cells)	Fluorimetry	Intracellular $[Ca^{2+}]$	Ketanserin
5-HT_{2A} Receptor (Agonist effect)	Human recombinant (HEK-293 cells)	Fluorimetry	Intracellular $[Ca^{2+}]$	Serotonin
5-HT_3 Receptor (Binding)	Human recombinant (CHO cells)	Scintillation counting	^{3}[H]BRL 43694	MDL 72222
CB_1 Receptor (Antagonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	AM 281
CB_1 Receptor (Agonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	CP 55940
D_1 Receptor (Agonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	Dopamine
D_1 Receptor (Antagonist effect)	Human recombinant (CHO cells)	HTRF	cAMP	SCH 23390
NMDA (Binding)	Rat cerebral cortex	Scintillation counting	^{3}[H]CGP 39653	CGS 19755
Glycine (strychnine-insensitive) (Binding)	Rat cerebral cortex	Scintillation counting	^{3}[H]MDL 105,519	Glycine
Adrenoreceptor-α-$_{2A}$ (Binding)	Human recombinant (CHO cells)	Scintillation counting	^{3}[H](-) Epinephrine	Epinephrine
$GABA_A$ (Binding)	Rat cerebral cortex	Scintillation counting	^{3}[H] muscimol	Muscimol
M_1 (Binding)	Human recombinant (CHO cells)	Scintillation counting	^{3}[H] Pirenzepine	Pirenzepine

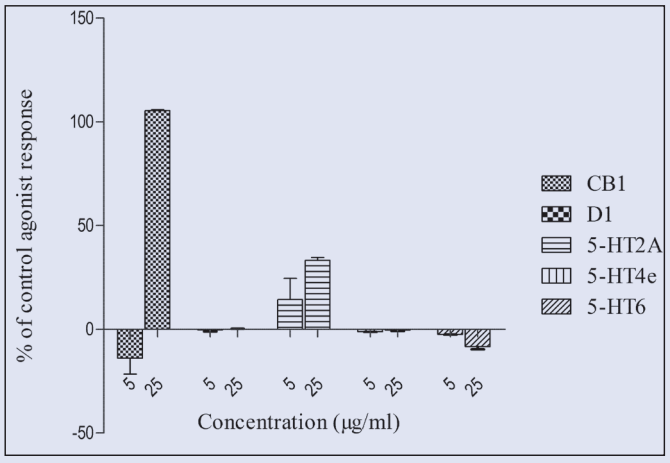

Figure 1: Agonist effect of BM on different receptors

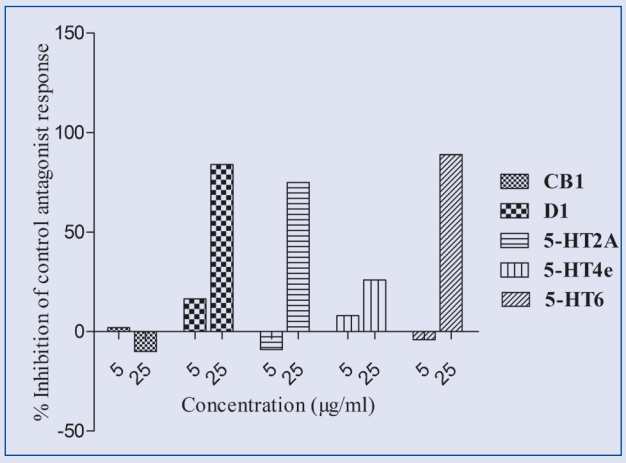

Figure 2: Antagonist effect of BM on different receptors

Table 2: Details of different enzyme based assays

Assay	Origin	Detection method	Product measured	Reference compound
β-Secretase (BACE-1)	Human recombinant (murine cells)	Fluorimetry	Mca-S-E-V-N-L-NH_2	OM 99-2
Phosphodiesterase 4D (PDE 4D)	Human recombinant (Sf9 cells)	HTRF	Residual cAMP	Rolipram
Protein Phosphatase (PP2B)	Bovine brain	Colorimetry	pNP	Trifluoperazine
Butrylcholinesterase (BuChE)	Equine serum	Colorimetry	2-nitro-5- mercapto-benzoate	Eserine hemisulfate
Acetylcholinesterase (AChE)	Electric eel	Colorimetry	2-nitro-5-mercapto-benzoate	Eserine hemisulfate
Monoacylglycerol Lipase (MGL)	Human recombinant	Colorimetry	2-nitro-5-thiobenzoate	Methyl arachidonyl fluoro-phosphate
Sirtuin 1 (SIRT 1)	Human recombinant	Fluorimetry	*Fluor de Lys*-SIRT 1 (Acetylated)	Resveratrol
Protein phosphatase1 (PP1)	Rabbit skeletal muscle (Recombinant enzyme)	Colorimetry	pNP	Okadaic acid
Oxidase-B Monoamine (MAO-B)	Human recombinant (Insect cells)	Luminescence	Luciferin	Pargyline HCl
11β-hydroxysteroid dehydrogenase type 1 (11-β HSD)	Human liver microsomes	HTRF	Cortisol d2	Carbenoxolone
Rho Kinase-II (ROCK-II)	Human recombinant (sf21 cells)	Colorimetry (ELISA)	Phosphorylated MBS (Myosin – binding subunit)	Y-27632
Proyl endopeptidase (PEP)	Rat brain	Fluorimetry	AMC	Z-Pro-Pro-CHO
Catechol-O-methyl transferase (COMT)	Rat liver	Fluorimetry	Scopoletin	3,5 dinitrocatechol
Insulin regulated aminopepetidase (IRAP)	Rat brain	Colorimetry	p-nitroanilide	Angiotensin IV
Lipoxygenase (LOX)	Soybean	Colorimetry	13-hydroxyperoxy linoleic acid	Indomethacin
Poly(ADP-ribose) polymerase-1 (PARP)	Human recombinant (*E.coli* cells)	Colorimetry	[(bio-ADP-ribose)n]	3-Amino-benzamide
Oxygen radical absorbance capacity (ORAC)	-	Fluorimetry	Sodium fluorescein	Trolox

assay was found to be 25.5 ± 7.32 µg/mL, 18.4 ± 1.28 µg/mL and 27.8 ± 0.73 µg/mL, respectively. The response of BM on other enzyme was not significant. The anti-oxidant activity of BM in ORAC assay was found to be 1698 µ moles TE/g. The inhibitory activity of BM in various assays is indicated in Tables 3 and 4.

DISCUSSION

In this study, we investigated the effect of BM on various molecular targets that are part of various neurological pathways like cholinergic, dopaminergic, cannabinoidergic, GABAergic and glutaminergic pathways, in order to elucidate its probable mechanisms of cognition enhancing activity.

Table 3: Median inhibitory concentration of BM in different enzymes inhibition assays

Assay	IC_{50}(µg/mL)
PEP inhibition	25.50 ± 7.32
COMT inhibition	18.40 ± 1.28
PARP-1 inhibition	27.80 ± 0.73
LOX inhibition	347.0 ± 0.70
MAO-B inhibition	367.2 ± 8.65
BuChE inhibition	713.4 ± 6.47

Table 4: Effect of BM on different enzymes targets

Assay	Concentration tested (µg/mL)	% Inhibition	Fold increase (compared to control)
ROCK-2 inhibition	100	22.42 ± 6 .14	
11-β HSD inhibition	150	30.96 ± 4.76	
AChE inhibition	2000	41.01 ± 0.04	
MGL inhibition	200	0.00 ± 0.00	
β-secretase	100	14.00 ± 0.68	
PP1 inhibition	200	29.89 ± 1.84	
PP2B inhibition	25	36.55 ± 0.64	
PDE 4D inhibition	25	4.55 ± 0.071	
IRAP inhibition	50	6.05 ± 2.19	
SIRT-1 activation	200	–	0.12 ± 0.04

BM was found to inhibit COMT, an enzyme which controls dopamine metabolism by methylation and thereby modulates memory functions. COMT inhibitors like entacapone, are used as adjuncts to levodopa in the treatment of Parkinson's disease.[14] Since, COMT inhibition has been associated with prefrontal cortex dopamine signaling, this enzyme forms an important component of dopaminergic signaling pathway.[15] Dopamine, which produces a stimulatory effect by acting on D1 receptors, stimulates the cAMP signaling pathway. BM could also be modulating the dopamine signaling pathway by inhibiting the activity of COMT enzyme. Bacosides from *B. monnieri* are reported to significantly increase the concentration of dopamine and serotonin in aged rat brains.[16] Thus, the potent inhibitory effect of BM on COMT enzyme could be corroborating its effect on memory and cognition via dopamine pathway. Studies have indicated that *B. monnieri* may prevent degeneration of dopaminergic neurons and increase the level of dopamine in cortex region of rat brain.[17] Even though BM does not directly affect the other molecular targets like D1 receptor itself or the MAO-B, PP1 enzymes of the dopamine signaling pathway, our studies indicate that it could be exerting a protective effect and enhancing the dopaminergic system by increasing the concentration of dopamine, a catecholamine required for long-term memory, by inhibiting the COMT enzyme.

The synthesis and release of neuropeptides arginine, vasopressin, substance-P, oxytocin and angiotensin II are known to strongly influence the learning and memory process.[18] BM, when tested for its inhibitory activity against two neuropeptidases; prolyl endopeptidase (PEP) and insulin-regulated aminopeptidase (IRAP), was found to inhibit the activity of PEP enzyme which is known to cleave short peptides with internal proline residues.[19] PEP degrades the neuropeptides arginine–vasopressin, oxytocin, neurotensin and substance-P that play a key role in positive reinforcement, social interactions, emotions and stress responsivity.[20] PEP has been acknowledged for its role in cognitive impairment.[21] Reduced PEP activity amplifies substance P-mediated stimulation of Ins $(1,4,5)P_3$ that stimulates the release of intracellular calcium, known to cause neurotransmitter release.[22] The effect of reduced PEP activity on calcium concentration is a novel intracellular function of this peptidase, that has an impact on the cognitive enhancement.[23]

BM exerted significant antagonist effect on 5-HT_6 receptors, which is an important component of serotonergic pathway. A growing body of evidence suggests the use of 5-HT_6 antagonists for treating cognitive dysfunctions[24] and BM exhibited significant antagonism at these receptors with an IC_{50} value of 52 ± 1.2 µg/mL. Also, 5-HT_6 receptor blockade has been reported to enhance cholinergic and glutaminergic neurotransmission[25] and increase dopamine level.[26] Thus, BM with an antagonistic effect on 5-HT_6 receptor could be affecting memory and cognition in more than one way. BM also had significant antagonistic effect on 5HT receptor subtype 2A. $5HT_{2A}$ receptor antagonists have been suggested to be beneficial for memory and cognition and have been implicated in insomnia[27] and also reported to increase sleep intensity. Morairty *et al.*[28] have presented the effect of sleep promotion via 5-HT_6 receptor blockade. So, the change in sleep intensity beacuse of $5HT_{2A}$ antagonism may improve cognition and vigilance during wakefulness.

Oxidative stress has been implicated in age associated memory disorders. Secondary injuries resulting from oxidative stress which produce DNA strand breaks that leads to cellular and neuronal death.[29] The ORAC value of BM was found to be 1698 µmoles TE/g suggesting its potential to scavenge the peroxyl radicals. It also inhibits the activity of PARP enzyme, which suggests that apart from reducing the free radicals generated, it can also reduce the secondary damage that has occurs due to free radical generation, adducing its potential as a good neuroprotective agent. The high ORAC value of BM is one of the reasons for its neuro-protection against oxidative stress.

The neurological pathways for memory have been reported to be overlapping.[30] The neural peptidergic pathway which includes arginine–vassopressin is augmented by the inhibition of enzymes in the dopaminergic pathway.[31] This indicates that inhibition of IRAP might be indirectly augmenting dopamine levels in the brain. The other pathways may also be linked through overlapping targets and thus modulation of one target may similarly affect other targets. Moreover, the metabolites of various Bacopa constituents could also be modulating these molecular targets for its memory enhancing activity as reported recently.[32]

CONCLUSIONS

The results of the present study demonstrate that BM exerts its effects on cholinergic, dopaminergic, and serotonergic pathways by affecting at least five different molecular targets of memory. The memory enhancing effects of BM can be linked to its cumulative effects on these enzyme and receptor based targets. Further, the reported clinical efficacy could also be on account of the pharmacological effect of metabolites generated as a result of intestinal and liver metabolism of the various phyto-constituents, on multiple targets implicated in memory and cognition, which warrants further studies to understand the overall mechanism of action of this herb.

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The authors declare that there is no conflict of interests regarding the publication of this paper.

Conflicts of interest

There are no conflicts of interest.

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