

# *Bacopa monniera* selectively attenuates suppressed Superoxide dismutase activity in Diazepam induced amnesic mice

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## KEY WORDS

Amnesia  
*Bacopa monniera*  
Diazepam  
L-NNA, MK 801, Scopolamine  
Superoxide dismutase

## ABSTRACT

**Background:** Amnesia is characterized by loss of memory that could result from abnormal neuro-chemical homeostasis, genetic predisposition or drug abuse. We earlier reported that *B. monniera* attenuates diazepam, scopolamine and L-NNA induced amnesia and wanted to test if SOD levels were affected by its administration. **Purpose:** *B. monniera* is earlier reported to augment the defense system for oxidative stress by increasing the activities of superoxide dismutase, therefore, we investigated its levels after *B. monniera* administration in combination with different amnesic agents. **Methods:** We treated mice with amnesic agents such as scopolamine, diazepam, L-NNA and MK 801 either with or without *B. monniera*. **Results:** Diazepam (1.75 mg/kg ip) significantly reduced SOD activity while it was unaltered when Scopolamine (0.1 mg/kg ip), MK 801 (0.17 mg/kg ip) and L-NNA (30 mg/kg ip) were administered. *B. monniera* significantly attenuated diazepam induced suppression of SOD activity. **Conclusion:** It is suggested that the mechanism of *B. monniera*'s anti-amnesic effect may vary depending on the type of amnesic agent used. However, antioxidant mechanism may be central to evoking the memory enhancing effects of *B. monniera* against diazepam induced amnesia.

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## Introduction

*Bacopa monniera* (Brahmi) is a popular herbal drug used worldwide. Its medicinal efficacy, is reported in Indian traditional literature such as *Athar-Ved*, *Charak Samhita*, *Susrutu Samhita*,<sup>1</sup> and is evidenced by various scientific studies in animals<sup>1-12</sup> and humans.<sup>13-21</sup> The major constituent of *Bacopa* was found to be bacoside-A and bacoside-B responsible for its cognition-facilitating effect. *B. monniera* in combination with *Ginkgo biloba* extract have shown improved anticholinesterase and antidementic activities in scopolamine induced amnesic mice.<sup>4</sup> *Bacopa monniera* and *Centella asiatica* are major constituents of 'Medhya rasayana', a Indian Ayurvedic herbal medicine. Herbex-kid, a polyherbal preparation containing *B. monniera* possess anti-allergic activity by 5-HT antagonism.<sup>22</sup> In addition to the anti-amnesic effects,<sup>6,7-9,12-23,24</sup> it exerts antioxidant,<sup>25</sup> antistress,<sup>26</sup> anxiolytic,<sup>27,28</sup> memory enhancing<sup>25</sup> and anti-ulcerogenic activities.<sup>29</sup> Recently it was reported that *B. monniera* also exerts anti-inflammatory<sup>30</sup> and anti-arthritis activity.<sup>31</sup> *Centella asiatica* is another herb reported to improve memory and promote the neuronal dendritic growth in hippocampus.<sup>32,33</sup> It also attenuates the spatial memory deficit in rat model of Alzheimer's disease.<sup>34</sup> Hosamani and Muralidhara have reported the neuro-protective efficacy of *Bacopa monnieri* against rotenone induced oxidative stress and neurotoxicity in *Drosophila melanogaster*.<sup>35</sup> Like animal studies, the clinical studies also provide equally robust evidence of *B. monniera*'s action on cognitive function. In a clinical study Sharma *et al* reported *B. monniera*'s effect on revitalizing the intellectual functions of children.<sup>36</sup> Some studies have suggested that the choice of dose and duration of *B. monniera*'s treatment as being critical for bringing its optimal effects. For example, chronic administration of 300 mg oral *B. monniera* for about 5-12 weeks substantially improves the higher order of cognitive processes in healthy humans.<sup>21</sup> Chronic administration of brahmi (*Bacopa*) decreases the rate of forgetting of newly acquired information in healthy humans.<sup>14</sup>

The pharmacological manipulation of LTP (long term potentiation) is useful in investigating the effect of various anti-amnesic drugs and their mechanisms. Scopolamine, an acetylcholine receptor antagonist impairs LTP and exerts amnesic effect on spatial learning and memory<sup>37,38</sup> when analysed by Morris water maze.<sup>39-41</sup> Diazepam, benzodiazepine receptor agonist, causes amnesia<sup>42,43</sup> and blocks long-term potentiation (LTP) in slices of hippocampus. When administered at chronic level (20 mg/kg/day, i.p.) for 21 days it induces anxiogenic reaction in mice.<sup>44</sup> It is, therefore, also useful in evaluation of anti-amnesic drugs.<sup>45,46</sup> MK 801 and L-NNA impair LTP by blocking NMDA receptor and inhibiting nitric oxide synthase enzyme respectively. MK 801 has been used as an amnesic agent for testing anti-amnesic effect of cannabidiol-rich extracts,<sup>47</sup> SB-399885 (a 5-HT<sub>6</sub> receptor blocker),<sup>48</sup> and thioperamide (a histamine-H<sub>3</sub> receptor antagonist).<sup>49</sup> Administration of 7-nitro-imidazole nitric oxide synthase (NOS) inhibitors have been reported to block hippocampal LTP and induce impairment of learning and memory in rats.<sup>50-52</sup> Aminoguanidine and L-N-iminoethyl-lysine, inducible NOS inhibitors, are also found to exacerbate the deficit in cognitive performance, as assessed by Morris water maze.<sup>51</sup> We earlier reported that *B. monniera* attenuates diazepam,<sup>7</sup> scopolamine<sup>53,54</sup> and L-NNA<sup>9</sup> induced amnesia. The biochemical analysis of mouse brain revealed the role of calmodulin, protein kinase and pCREB in bringing about the anti-amnesic effects of *B. monniera*. As Superoxide dismutase (SOD) has earlier been reported to be responsible for detoxification for free radicals we wanted to test if *B. monniera* was able to augment an anti-oxidative defence system<sup>6-25-55-59</sup> by increasing the activity of superoxide dismutase.<sup>56-58-61</sup>

## Methods

### Animal

Swiss albino mice (male, age matched, weight 25-35 g) were used in the study and housed four per cage with *ad libitum*

access to food and water under controlled laboratory conditions. Experiments were conducted between 9.00 to 18.00 hrs in a semi-sound proof laboratory. All experiments were performed in accordance with the guidelines of Institute animal ethical committee and European Communities Council Directive (86/609/EEC). Adequate measures were taken to minimize pain or discomfort with animal experimental procedures.

#### Drugs and Chemicals

*Bacopa monniera* (brahmi) standardized extract, containing 55.34% of bacosides, was obtained from Lumen marketing company, Chennai. The standardized extract of *B. monniera* suspended in Tween 80 (5% v/v in normal saline) and scopolamine, L-NNA and MK801 (Sigma Aldrich, USA) were dissolved in normal saline.

#### Drug Treatment Schedule

Mice in Group I were administered normal saline (10 ml kg<sup>-1</sup>) orally for 6 days. Group II mice were injected with 5% Tween 80 (10 ml kg<sup>-1</sup>) orally and normal saline (10 ml kg<sup>-1</sup> ip) with a gap of 30 min. Group III to VI mice were treated with Tween-80 (10 ml kg<sup>-1</sup>, orally) and test amnesic agent. We used four amnesic agents separately: L-NNA (30 mg kg<sup>-1</sup> i.p.), MK 801 (0.17 mg

kg<sup>-1</sup> i.p.), Scopolamine (0.1 mg kg<sup>-1</sup> i.p.) and Diazepam (1.75 mg kg<sup>-1</sup> i.p.). Group VII mice were administered standardized extract of *B. monniera* (80 mg kg<sup>-1</sup> oral) and L-NNA (30 mg kg<sup>-1</sup> i.p.) at 30 min of time interval. Group VIII-X mice were administered *B. monniera* (120 mg kg<sup>-1</sup> oral) and MK 801 (0.17 mg kg<sup>-1</sup> i.p.) / Scopolamine (0.1 mg kg<sup>-1</sup> i.p.) / Diazepam (0.1 mg kg<sup>-1</sup> i.p.) at similar time interval. We also studied *per-se* effect of *B. monniera* (120 mg kg<sup>-1</sup>).

#### Superoxide Dismutase

After six days the mice were sacrificed by cervical dislocation. Isolated brain was frozen for biochemical estimation. Brain homogenate and the supernatant were preserved for future analysis. SOD level was measured by superoxide dismutase estimation kit (Sigma, USA). The standard protocol available with the kit was used for estimating SOD, which was further normalized by total protein. The total protein was estimated by Bradford method.

The biochemical results were analyzed by ANOVA followed by post hoc tests such as least significance difference (LSD). 'a' indicates significance at  $p < 0.05$  of treated group versus control group. 'b' indicates significance at  $p < 0.05$  of treated group versus amnesic (scopolamine/diazepam/L-NNA/MK 801) group.

**Table 1: Drug Treatment Schedule**

No.	Group	Treatment	Dose and Duration
I	Control	Normal Saline	10 ml kg <sup>-1</sup> orally, for 6 days
II	T80	5% Tween 80 and Normal Saline	T80: 10 ml kg <sup>-1</sup> orally NS: 10 ml kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
III	L-NNA	5% Tween 80 and L-NNA	T80: 10 ml kg <sup>-1</sup> orally L-NNA: 30 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
IV	MK	5% Tween 80 and MK 801	T80: 10 ml kg <sup>-1</sup> orally MK: 0.17 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
V	Sco	5% Tween 80 and Scopolamine	T80: 10 ml kg <sup>-1</sup> orally Sco: 0.1 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
VI	DZ	5% Tween 80 and Diazepam	T80: 10 ml kg <sup>-1</sup> orally DZ: 1.75 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
VII	BM + L-NNA	<i>B. monniera</i> and L-NNA	BM: 80 mg kg <sup>-1</sup> orally L-NNA: 30 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
VIII	BM + MK	<i>B. monniera</i> and MK 801	BM: 120 mg kg <sup>-1</sup> orally MK: 0.17 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
IX	BM + Sco	<i>B. monniera</i> and Scopolamine	BM: 120 mg kg <sup>-1</sup> orally Sco: 0.1 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
X	BM + DZ	<i>B. monniera</i> and Diazepam	BM: 120 mg kg <sup>-1</sup> orally DZ: 0.1 mg kg <sup>-1</sup> i.p. with a gap of 30 min, for 6 days
XI	BM	<i>B. monniera</i>	120 mg kg <sup>-1</sup> orally, for 6 days

## Results

### *B. monniera* exerts antioxidative effects by attenuating diazepam induced suppression of superoxide dismutase

SOD activity was reduced with diazepam treatment of mice as compared to control mice. *B. monniera* alleviated the suppressed SOD activity when *B. monniera* was administered with diazepam when compared to diazepam treated mice. It suggests that an antioxidant mechanism might play an important role in the reversal of diazepam induced amnesia. Tween 80, used as a vehicle to prepare the suspension of *B. monniera* did not alter the SOD activity as compared to control mice. Similarly *B. monniera* alone did not affect the SOD activity as compared to control mice (Fig 1A).

### Endogenous antioxidative defense mechanism is unaltered with *B. monniera* in L-NNA treated mice

SOD activity was not significantly affected by L-NNA treatment as compared to control mice. Similarly *B. monniera* did not affect SOD activity in L-NNA pretreated group (Fig 1B).

### Scopolamine and MK 801 alone do not, but along with *B. monniera* reduce antioxidative effect in mice

*B. monniera*, when administered with scopolamine, partially reduced the SOD activity, but when scopolamine was administered alone, it did not alter the SOD activity significantly as

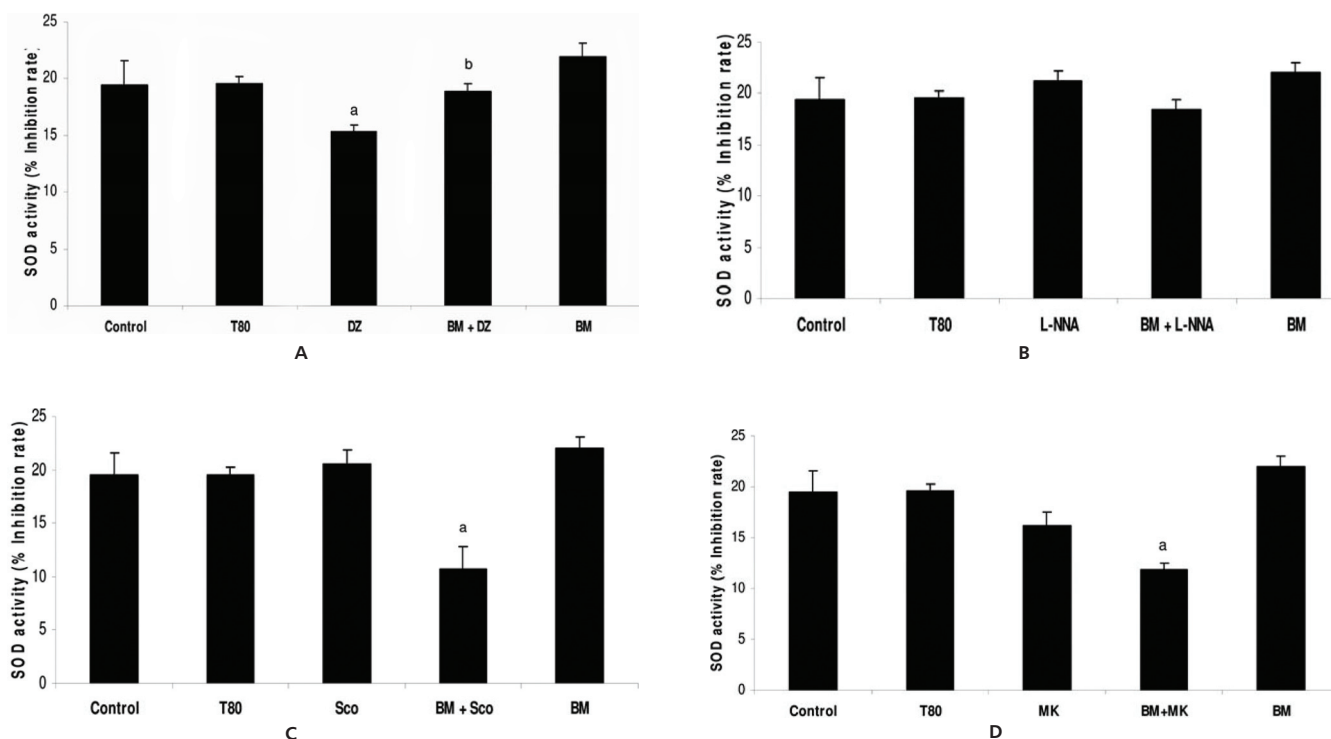
compared to control mice (Fig 1C). Similarly, *B. monniera* and Tween 80 *per se* did not affect SOD activity.

SOD activity was not significantly reduced with MK 801 treatment in mice as compared to control mice. It was further suppressed by pre treatment of *B. monniera* with MK 801 (Fig 1D).

## Discussion

This preliminary study demonstrates that *B. monniera* extract enhances the learning ability of rats.<sup>62</sup> *B. monniera* is known to reduce the level of amyloid especially Abeta 1-40 and 1-42 in doubly transgenic mouse model of Alzheimer's Disease.<sup>63</sup> Subsequent studies have indicated that cognition-facilitating effect of standardized extract of *B. monniera* has been due to two prominent constituents, bacoside-A<sup>64,65</sup> and bacoside-B.<sup>65,66</sup> Another active constituent of *B. monniera*, Betulinic acid, attenuates interleukin-6 production and exerts anti-inflammatory effect.<sup>30</sup> *B. monniera* promotes cell survival in response to oxidative stress by suppressing the formation of reactive oxygen species and any change in the activity of redox regulated proteins, i.e., NF-kappaB, Sirt1, ERK1/2, and p66Shc involved in the pathophysiology of Alzheimer's Disease.<sup>67</sup>

When *B. monniera* was administered as adjunct it was shown to improve the beneficial effect of *ginkgo biloba* on cognition deficits<sup>2</sup> besides reducing the side effects (i.e. cognition deficit) of Phenytoin.<sup>24</sup> Combined with these studies, we additionally

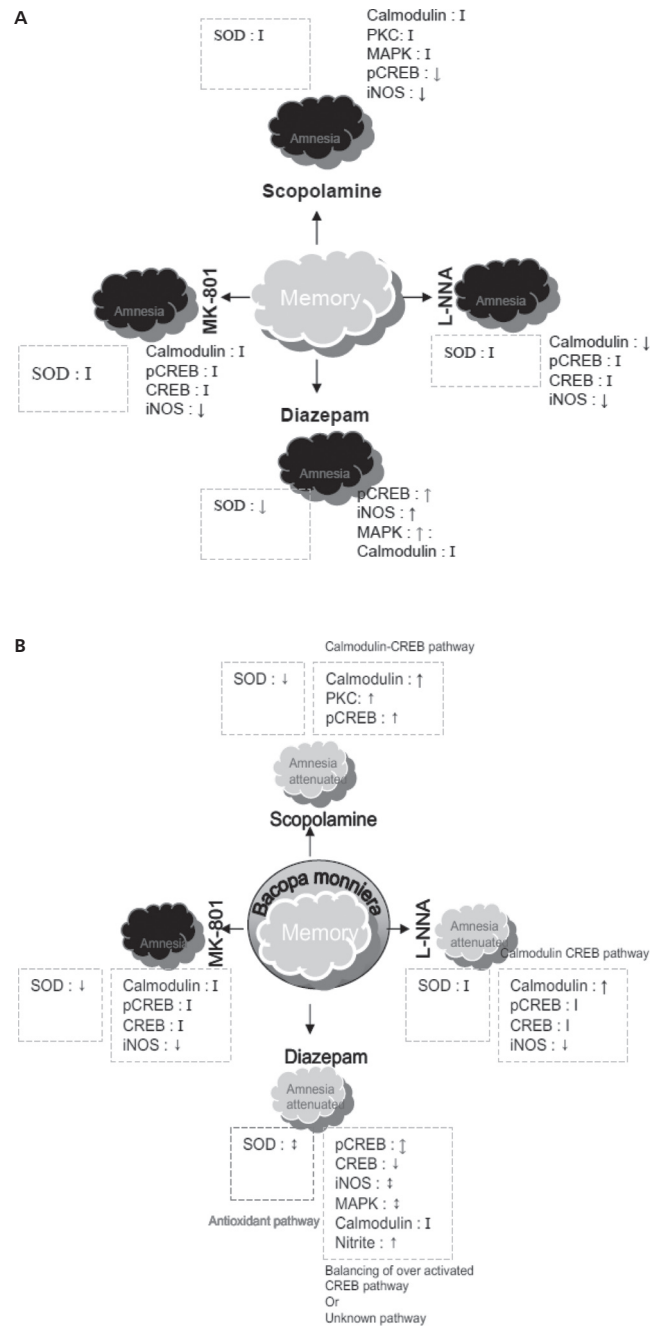


**Fig. 1:** *Bacopa monniera* selectively attenuates suppression of Superoxide Dismutase activity. 5% Tween 80 and *B. monniera* (120 mg/kg oral) did not alter SOD activity. Diazepam 1.75 mg/kg ip (fig 1A) and MK 801 (0.17 mg/kg ip (fig 1D) significantly reduced SOD activity, while Scopolamine 0.1 mg/kg ip (C) and L-NNA 30 mg/kg ip (fig 1B) could not change it. *B. monniera* significantly attenuated diazepam induced suppression of SOD activity (fig 1A). On the other hand *B. monniera* did not produce significant impact of L-NNA (fig 1B) and MK 801 (fig 1D) pretreated mice, while it further suppressed SOD activity in Scopolamine pretreated mice (fig 1C). These values were obtained after normalization with total protein. Data was analyzed by ANOVA followed by LSD test. 's' indicates significant difference for treated group vs control group at  $p < 0.05$ , 'b' indicates significant difference for treated group vs amnesic agent (diazepam/ scopolamine/L-NNA/MK 801) group at  $p < 0.05$ .

reported that *B. monniera* significantly reverses diazepam,<sup>7,11</sup> scopolamine<sup>23-53</sup> and L-NNA induced amnesia but not MK801 induced amnesia.<sup>9</sup> In order to correlate the behavioural results for understanding the intracellular molecular pathway we investigated *B. monniera*'s effect on various downstream molecules and enzymes in amnesic mice brains. We have shown in our earlier studies that scopolamine downregulates protein kinase C and iNOS but it does not affect cAMP, protein kinase A, calmodulin, MAP kinase, nitrite, CREB and pCREB. *B. monniera* reverses the scopolamine induced amnesia by significantly improving calmodulin and partially attenuating protein kinase C and pCREB.<sup>23</sup> Moreover, we also found that *B. monniera* increases calmodulin (CaM) and pCREB/CREB levels when L-NNA was used as amnesic agent. We did not find alteration in cAMP, PDE, nitrate, nitrite, iNOS and total CREB levels in L-NNA or MK 801 treated mice (data not shown). Diazepam upregulates MAP kinase, pCREB and iNOS, while it downregulates nitrite, nitrate, total nitrite, CREB expression, phosphodiesterase, cAMP without affecting calmodulin levels. *Bacopa monniera* also suppressed the diazepam induced upregulation of MAP kinase, pCREB and iNOS and attenuated the downregulation of nitrite. It, however, does not affect the cAMP, PDE, nitrate, total nitrite, total CREB level.<sup>12</sup>

Since *B. monniera* has a differential anti-amnesic effect which can not be explained by a universal pathway, we analysed SOD for anti-amnesic effect of *B. monniera*. We found that the level of SOD was significantly reduced with diazepam and partially reduced with MK 801 treatment, but it was not affected by scopolamine and L-NNA treatment. *B. monniera* alleviated the SOD activity when *B. monniera* was administered with diazepam. It suggests that the antioxidant mechanism plays dominant role in reversing diazepam induced amnesia. To support our hypothesis, we report El-Sokkary's findings that antioxidants like melatonin and vitamin C could restore the levels of superoxide dismutase (SOD) activity and glutathione (GSH) concentration in liver tissues of rats administered with diazepam.<sup>68</sup>

*B. monniera* did not attenuate the SOD activity in the L-NNA group. On the other hand *B. monniera* partially reduced the SOD activity in scopolamine pretreated mice and MK801 pretreated group (Fig 1A-D). We earlier found that total nitrite was not much affected by scopolamine alone while the combination of scopolamine and *B. monniera* suppresses the total nitrite. We assume that the superoxide dismutase enzyme was consumed for deactivation of the free radicals due to formation of nitric oxide metabolites. It suggests that the antioxidant mechanism participates indirectly in association with other mechanism for reversal of scopolamine induced amnesia. Along with the evidence from our previous studies<sup>11,12-23</sup> we can conclude that the mechanism of *B. monniera*'s anti-amnesic effect is different for diazepam than that for scopolamine. However, antioxidant mechanism may contribute towards anti-amnesic effects of *B. monniera* against diazepam induced amnesia (Fig 2). *B. monniera* is shown to improve the cognitive deficit possibly by exhibiting free radical scavenging and anti-lipid peroxidative effects<sup>58</sup> that protect the brain from oxidative damage, and by augmenting the anti-oxidative defence system of glutathione, vitamin C, vitamin E, and vitamin A along with the activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and glutathione reductase (GR) while maintaining the levels of trace elements such as copper, iron, zinc and selenium.<sup>58</sup>



**Fig. 2:** A hypothetical representation of mechanism of *Bacopa monniera*. (A) Scopolamine, Diazepam, L-NNA and MK 801 impair the memory and produce amnesia. Scopolamine, diazepam and L-NNA downregulate (or do not change) intracellular messenger molecules such as Calmodulin, pCREB, iNOS etc. On the other hand, Diazepam upregulates most of these molecules. (B) *Bacopa monniera* protects the brain from exposure of these drugs and attenuates the amnesia produced by scopolamine, diazepam and L-NNA. But it does not revert the amnesia induced by MK801. *Bacopa monniera* attenuates the SOD activity and balances the over activation of CaM - CREB pathway which indicates important role of antioxidant pathway over CaM-CREB pathway. However CaM - CREB pathway plays a substantial role for reversal of scopolamine and L-NNA induced amnesia by *Bacopa monniera*. MK801 treatment does not affect either Cal-CREB pathway or antioxidant pathway.



*B. monniera* and Tween 80 *per se* did not affect SOD activity (Fig 1A-D). In earlier behavioral studies with Morris water maze we were not able to find any significant effect of *B. monniera per-se* on normal acquisition and retrieval of memory.<sup>7-11,23-53</sup>

### Conclusion

On the basis of our findings we conclude that *B. monniera* extract possesses antioxidant activities that are possibly mediated by SOD. However, additional studies with the use of antioxidants as controls managing the homeostasis of antioxidant profile in brain tissue can further make such studies more valuable.

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