Ocimum Sanctum Linn: A Potential Adjunct Therapy for Hyperhomocysteinemia-Induced Vascular Dementia

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1 Introduction

Dementia a common and collective term for progressive loss in cognitive and intellectual functioning. According to WHO statistics 35.6 million people were known to suffer from dementia, worldwide in 2010. This number was expected to increase by almost twofold every 20 years, i.e., to 65.7 million in 2030 and 115.4 million in 2050 (Prince et al. 2013). Vascular dementia (VaD) is second most common form of dementia after Alzhiemer's dementia. Vad occurs as a result of ischemic or hemorrhagic insult's that injure innermost regions of the brain such as the cortex or the hippocampus which play an important role in functions like memory, cognition,

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and behavior and functional abilities (Ekonomou et al. 2011; Román 2003). Acute oral administration of L-methionine or subcutaneous administration of DL-homocysteine in rats induce hyperhomocysteinemia (HHcy), which further leads to conditions like vascular dementia (VaD) (Hemanth Kumar et al. 2016, 2017; Boyina et al. 2018). Various studies suggest that there is a positive dose-dependent relationship between the increase in plasma total Hcy concentrations and neurodegenerative diseases like VaD and stroke (Nilsson et al. 2010; Obeid and Herrmann 2006). Serum homocysteine levels more than 15 μM are termed as HHcy (Seshadri et al. 2002). HHcy was previously reported for inhibiting the expression of antioxidant enzymes, and causing complex changes in blood vessels that include endothelial dysfunction (ED), oxidative stress, and pro-inflammatory effects such as expression of tumor necrosis factor (TNF) and inducible nitric oxide (NO) synthase (Hankey and Eikelboom 1999).

Ocimum sanctum (OS) (Lamiaceae) generally known as "holy basil," commonly found in India, is used as an important component in Ayurveda for the treatment of skin diseases, asthma, malaria, dysentery, chronic fever, hepatic diseases, and microbial infections. Previous literature on *Ocimum sanctum* reveals that leaves contain various bioactive constituents such as flavonoids and essential oils. These active constituents are known for their diverse biological activities and are responsible for neuroprotective effects against hypoperfusion-induced cognitive deficits and ischemia-induced oxidative stress (Kelm et al. 2000; Yanpallewar et al. 2004). Vascular dementia is known to be associated with cognitive deficits, oxidative stress, and endothelial dysfunction, but there are no reports suggesting the neuroprotective role of OS in VaD animal models. So, the present study was designed to evaluate the potential effect of ethanolic extract of *Ocimum sanctum* (EEOS) as an adjunct therapy for hyp0 erhomocysteinemia-induced vascular dementia and oxidative stress in rats and to predict the active chemical constituents responsible for this potential effect.

2 Materials and Methods

Male Wistar rats weighing 150–200 g were used in the present study. All animals were maintained under standard husbandry conditions. The rats were randomly assigned into six groups ($n = 6$). The experimental protocol was duly approved by the Institutional Animal Ethics Committee (Reg. No, I/IAEC/LCP/027/2013/WR-36). Fresh leaves of *Ocimum sanctum* were collected locally and were dried under shade and powdered, and the leaf ethanolic extract was prepared. This method involves extraction by percolation at room temperature using 70% ethanol. The yield of the final product during the extraction procedure was 17.32% (w/w). L-methionine (1.7 g/kg/day, p.o.) was administered for 4 weeks to induce hyperhomocysteinemia-associated vascular dementia and endothelial dysfunction in rats (Hemanth Kumar et al. 2016, 2017). EEOS, 100 mg/kg; EEOS, 200 mg/kg; EEOS, 400 mg/kg; and donepezil, 0.1 mg/kg p.o. were administered to L-methionine

(1.7 g/kg/p.o.) treated animals starting from the 14th day to 32nd day of the study. Animal grouping and experimental design were shown in Table 1 and Fig. 1, respectively. The spatial learning and working memory of all animals were assessed by Y-maze and Morris water maze (MWM) tasks (Hemanth Kumar et al. 2016, 2017). Y-maze was used for assessing spatial working memory and MWM test is used to assess hippocampal-dependent learning and memory. In MWM test an acquisition trial was conducted from 28th to the 31st day of the study followed by a retrieval trial on the 32nd day by using a video tracking system. After behavioral analysis, blood samples were collected for the estimation of serum biochemical parameters. Serum Hcy was determined according to the method described earlier using chemiluminescent microparticle immunoassay (Kemse et al. 2014) (Abbott Laboratory, Abbott Park, Chicago, IL). Serum nitrite was determined using the earlier described

Table 1 Grouping of animals

Animal groups	Treatment
Group I	0.5% carboxymethyl cellulose (10 ml/kg, p.o.) for 32 days
Group II	L-methionine (1.7 g/kg/p.o.) for 32 days
Group III	L-methionine (1.7 g/kg/p.o.) + EEOS low dose (100 mg/kg, p.o.) from 14th day to 32nd day
Group IV	L-methionine (1.7 g/kg/p.o.) + EEOS mid dose (200 mg/kg, p.o.) from 14th day to 32nd day
Group V	L-methionine (1.7 g/kg/p.o.) + EEOS high dose (400 mg/kg, p.o.) from 14th day to 32nd day
Group VI	L-methionine (1.7 g/kg/p.o.) + Donepezil (0.1 mg/kg, p.o.) from 14th day to 32nd day

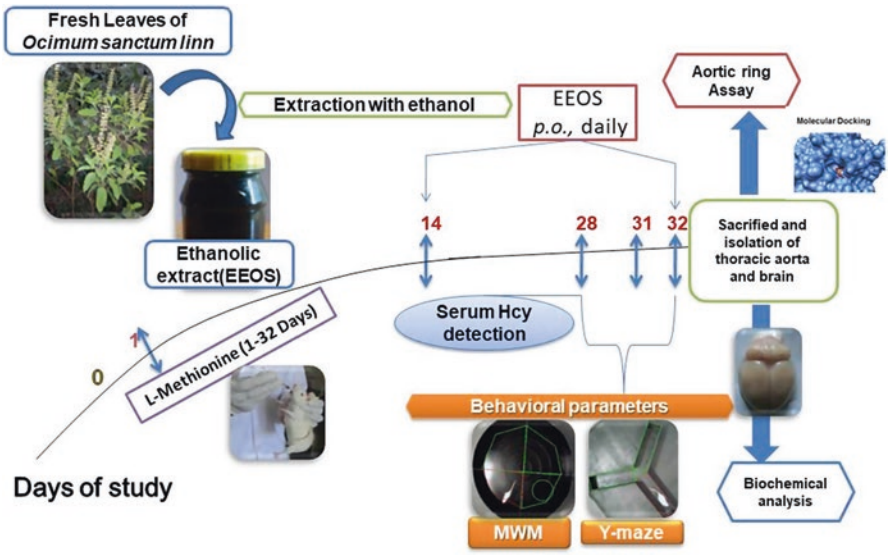


Fig. 1 Experimental Design

method of Sastry et al. (2002) with slight modifications. Rats with serum homocysteine levels of >15 μM were considered to be hyperhomocysteinemic. All animals were later sacrificed under anesthesia for isolation of thoracic aorta and brain tissues for biochemical estimations. Thoracic aorta was used for aortic ring assay, (Joseph and Nair 2013) and the clear supernatant of brain homogenate obtained was used for estimation of brain biochemical parameters like brain acetylcholinesterase, lipid peroxidation, reduced glutathione, and superoxide dismutase (SOD) levels. All biochemical procedures were carried out according to our previously published studies.

(Vinitha et al. 2014, Hemanth Kumar et al. 2016, 2017) All results were mentioned as mean ± SEM and analyzed using one-way ANOVA, followed by Tukey's multiple range tests. The results for aortic ring preparation were statistically analyzed by using repeated measure analysis of variance (ANOVA), followed by Newman-Keuls test. The statistical significance of difference was taken as $P < 0.05$. All the study results were statistically analyzed using GraphPad Prism 5.0 software.

At the end of this study, molecular docking studies were carried out for *Ocimum sanctum* leaf active constituents,(Joseph and Nair 2013) viz., apigenin, ascorbic acid, β-carotene, β-caryophyllene, luteolin, eugenol, carvacrol, methyl eugenol, Molludistin, and orientin. These chemical constituents were selected for docking in order to predict the possible binding interactions with S-adenosyl homocysteine hydrolase (SAH), which is an enzyme responsible for the metabolism of methionine (Obeid and Herrmann 2009). The crystal structure of SAH (PDB: 1LI4) was retrieved from the protein data bank (http://www.rcsb.com). The docking was carried out using default settings of the MOE program by initially preparing ligand and protein before docking (Radhakrishnam et al. 2019).

3 Results and Discussion

Plant polyphenols play a key role in controlling the levels of reactive oxygen species (ROS) and thus help in maintaining normal cell function. Considerable epidemiological data has been collected to recommend an association between consumption of fruits or leaves containing antioxidants and a reduced risk of certain chronic diseases (Kaur and Kapoor 2001; Pandey and Rizvi 2009). For the first time, this study reveals the neuroprotective effect of EEOS on hyperhomocysteinemia-induced vascular dementia and oxidative stress in rats. The MWM and Y-maze tests used in the current study were one of the generally accepted behavioral models for assessment of spatial learning memory ([22]. The percentage alternation of L-methionine-treated group (Group II) was significantly ($P < 0.001$) lowered and prevented L-methionine-induced decrease in percentage alternations compared with L-methionine-treated group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, and $p < 0.001$) (Fig. 2a). The change in the behavior of rats in Y-maze indicates an assessment of spatial working or short-term memory.23.

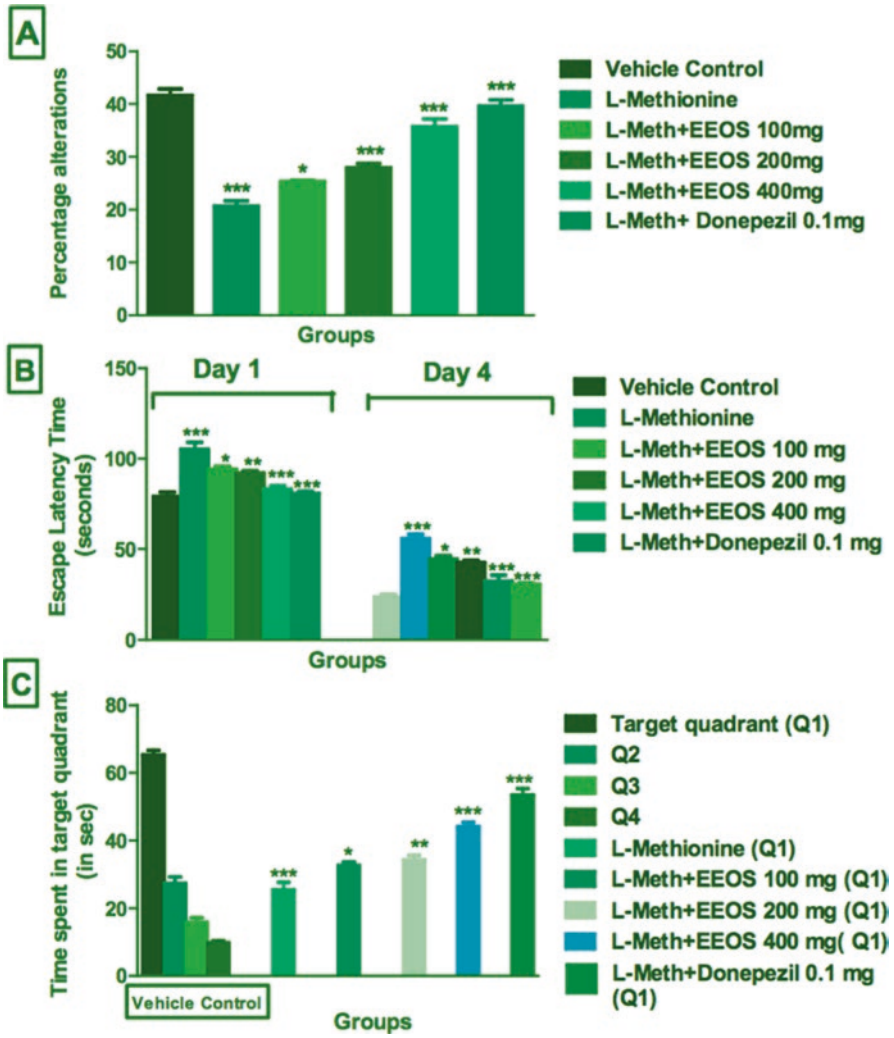


Fig. 2 Effect of EEOS on Y-maze test performance and ELT and TSTQ of Morris water maze test. All the results are expressed as mean ± SEM of $n = 6$ animals. The significance was defined as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; comparison of treated groups, control groups, and negative control groups

In the current study during Y-maze test, rats showed a decline in percentage alternations, which reveals the index of behavioral neurotoxicity after the administration of L-methionine. The main mechanism behind this could be due to HHcy and its effect on impaired function of N-methyl-D-aspartate receptor (NMDA) mediated through glutamate-NO/cyclic GMP pathway in the hippocampus, which is also known to be involved in the various forms of cognition and memory function (Obeid and Herrmann 2006). A significant increase in the percentage of alternation

in drug-treated animals during the Y-maze test was observed indicating EEOS role in the improvement of spatial working memory. These results were in concurrence with the results derived from testing of various natural dietary antioxidants and plant extracts published from our lab.

In the MWM test, the vehicle control-treated rats showed a decreased tendency in ELT of MWM. There was a significant fall in day 4 ELT when compared to day 1 ELT of these rats ($p < 0.001$), indicating their normal learning ability. In addition, on day 5, a significant increase in TSTQ was noted, when compared to the time spent in other quadrants ($p < 0.001$) that indicates a normal retrieval. However, L-methionine-treated rats showed a significant increase in day 4 ELT when compared to day 4 ELT of vehicle control animals ($p < 0.001$), also indicating impairment of acquisition. However, L-methionine administration also produced a significant decrease in day 5 TSTQ when compared to day 5 TSTQ of vehicle control ($p < 0.001$; indicating impairment of memory as well). Administration of EEOS (low, mid, and high dose) and donepezil significantly prevented L-methionine-induced rise in day 4 ELT ($P < 0.05$, $P < 0.01$, and $P < 0.001$) and $P < 0.001$, indicating reversal of L-methionine-induced impairment of acquisition. Further treatment also attenuated L-methionine-induced decrease in day 5 TSTQ in a significant manner ($P < 0.05$, $P < 0.01$ and $P < 0.001$, and $P < 0.001$), indicating a reversal of L-methionine-induced impairment of memory (Fig. 2b, c). The significant fall in escape latency time (ELT day 4) of control animals during acquisition trials represents the normal acquisition of memory. An increase in time spent in the target quadrant (TSTQ), in search of the invisible platform during retrieval, represents improvement of memory. In this present study, EEOS showed a significant effect in spatial learning and memory which was evident from the increase in the time spent in the target quadrant and the decrease in escape latency time on MWM, compared to L-methionine-treated rats. Treatment with three different doses of EEOS and donepezil had improved spatial recognition memory as evidenced by improved performance in the Morris water maze and the Y-maze. These results were in concurrence with the results derived from testing of various natural dietary antioxidants and plant extracts published from our lab (Hemanth Kumar et al. 2016, 2017; Boyina et al. 2018; Singh et al. 2015; Vinitha et al. 2014). HHcy has been shown to induce endothelial dysfunction by decreasing the bioavailability of nitric oxide (NO) and development of vascular oxidative stress. The reduced NO level has been shown to contribute to the pathogenesis of vascular dementia. Consequently, the serum nitrite concentration has been selected as a definitive marker of endothelial dysfunction (Abahji et al. 2007). In the current study, endothelial dysfunction was assessed by measuring the relaxation response of acetylcholine and sodium nitroprusside on precontracted aortic ring preparation of rats from different groups.

In L-methionine-treated rats, we observed a significant decline in acetylcholine-induced endothelium-dependent relaxation; however, SNP-induced relaxation was similar in animals of all groups, indicating that the capacity of the vascular smooth muscle to relax in response to exogenous NO was not impaired. Moreover, we observed an increase in vasoconstrictor responses to phenylephrine, and the mag-

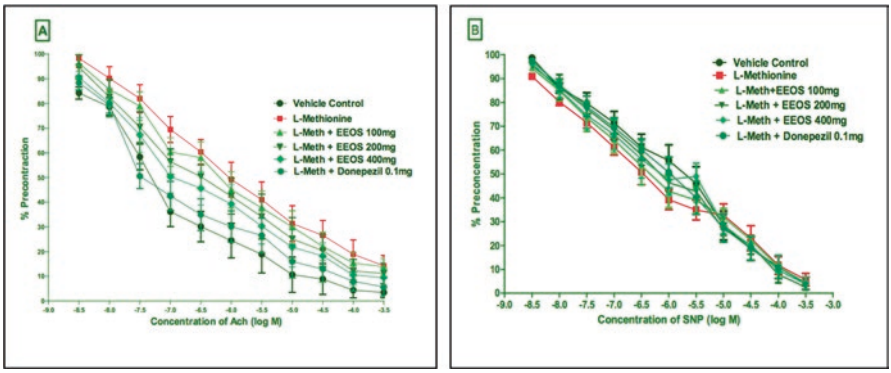


Fig. 3 Effect of EEOS on acetylcholine-induced endothelium-dependent relaxation and SNP-induced endothelium-independent relaxation. All the results are expressed as mean ± SEM of $n = 6$ animals. Responses are expressed as a percentage of precontraction induced by 3×10^{-6} M phenylephrine. The repeated measure (ANOVA) was performed, followed by Newman-Keuls test. (**a**) $p < 0.05$ versus vehicle control, (**b**) $p < 0.01$ versus L-methionine-treated group

nitude of the response to phenylephrine was lower in the aortic rings of L-methionine-treated rats. Compared with the vehicle control rats, the endothelium-dependent relaxation, induced by acetylcholine, was strong ($p < 0.05$), and a significant decrease in the negative control group (L-methionine) was noticed (Fig. 3a). However; it did not affect SNP-induced endothelium-independent relaxation (Fig. 3b). This dysfunction in the aorta was improved by treatment with EEOS (low, mid, and high dose), and donepezil administration significantly prevented the effect of L-methionine on endothelial relaxation ($p < 0.01$). Hence, our results recommend that the increased vascular reactivity to phenylephrine and the relative reduction of endothelial changes associated with decreased acetylcholine reactivity suggest that the treatment with L-methionine reduces NO bioavailability. EEOS demonstrated a protective role on endothelium by increasing the bioavailability of NO in a blood vessel and by decreasing the serum homocysteine levels, resulting in the improvement of the vascular dementia condition. Excess reactive oxygen species cause cell injury by damaging lipids, proteins, and DNA in the cell (Mattson and Shea 2003). Although an association between HHcy and oxidative stress has been reported, the mechanisms by which Hcy causes VaD remain poorly understood (Yang et al. 2003). In the present study, administration of L-methionine produced a significant increase in serum Hcy, brain TBARS, and depleted reduced glutathione, SOD, and catalase activities, suggesting oxidative damage. Treatment with EEOS (low, mid, and high dose) and donepezil has shown a significant effect on L-methionine and significantly reduced AChE and TBARS and attenuated the fall in SOD, GSH, serum Hcy, and nitrite levels in Group III, IV, V, and VI when compared with L-methionine-treated group (Group II) ($p < 0.05$, $p < 0.01$ and $p < 0.001$, and $p < 0.001$) (Table 2) demonstrating their antioxidant-like effect which is in

concurrence with our previous reports on L-methionine-induced HHcy (Hemanth Kumar et al. 2016, 2017).

Acetylcholinesterase inhibitors are the major class of drugs which are often used for the treatment of cognitive impairment. Donepezil is a cholinesterase inhibitor which is commonly used in the management of Alzheimer's disease (AD). Donepezil is known to show its therapeutic action by inhibiting AChE enzyme, thereby provoking a rise in Ach levels in the synapse. Donepezil also demonstrated to have antioxidative and neuroprotective actions (Koladiya et al. 2008). EEOS and donepezil at different doses show their neuroprotective effect by decreasing the raised levels of brain AChE.

We tried to predict the potential of *Ocimum sanctum* by docking its active chemical leaf constituents into the SAH active site, using the docking tool of MOE. The X-ray crystallographic structure of SAH complexed with neplanocin (PDB: 1LI4) was used for the docking calculations (Yang et al. 2015). The protein structure was optimized using structure preparation, and energy minimization was carried out using MOE default settings. The active site was determined using the "Site Finder" tool of the program. The binding free energy dG and the ligand-protein interactions with distance and energy were calculated and are shown in Table 3. The energy data showed that molludistin (IX) exhibited highest binding energy (dG) against SAH. The most active compounds IX and VII showed the highest dG values of −8.98 and − 7.08 kcal/mol, respectively, whereas crystal ligand has shown lesser binding energy with dG value −8.68 kcal/mol compared with IX. The results of 2D and 3D interactions were shown in Fig. 4. By this molecular docking study, it indicates that molludistin (IX) could be a specific chemical constituent for treatment as an adjunct therapy against hyperhomocysteinemia-induced vascular dementia. A further study is needed to explore the mechanism of molludistin against HHcy. EEOS in various studies has been reported to exert antioxidative, potential anti-neuroinflammatory actions and have neuroprotective and cognition-enhancing activity (Giridharan et al. 2011; Sampath et al. 2015; Suanarunsawat et al. 2009). Therefore, with support from earlier research and our study data, it may be proposed that EEOS mediates neuroprotective effect in L-methionine-induced vascular dementia and oxidative stress, which is due to its multiple effects including antioxidant, anticholinesterase, and memory-enhancing activity.

In conclusion, results show that the EEOS possess neuroprotective effect on HHcy-induced neurotoxicity and VaD. EEOS has incredibly restored cognitive impairments, learning, and memory. It also diminished the levels of AChE and has shown the protective role of endothelium by increasing the bioavailability of NO, thus decreasing the vascular oxidative stress. Further research on EEOS and its active chemical constituents especially flavonoids like molludistin is needed, for the lucid understanding of its molecular mechanism and in order to elucidate the important pathways that play a pivotal role in the process of neurodegeneration and vascular dysfunction.

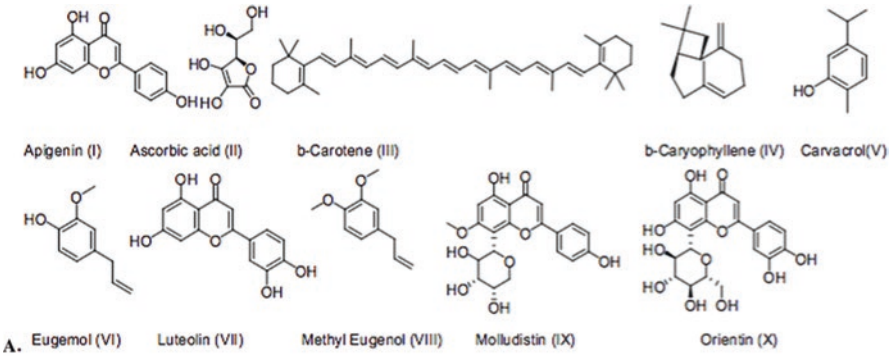
Table 2 Effect of EEOS on brain and serum biochemical parameters

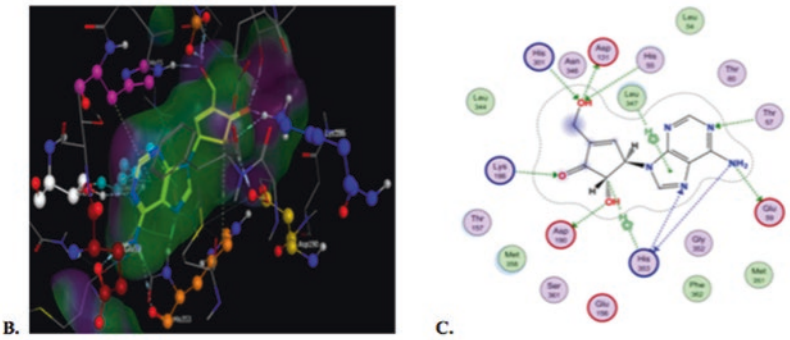
Biochemical parameters	Vehicle control	L-Methionine (1.7 g/kg)	L-Meth + EEOS (100 mg/kg)	L-Meth + EEOS (200 mg/kg)	L-Meth + EEOS (400 mg/kg)	L-Meth + donepezil (0.1 mg/kg)
Brain AchE (u/mg protein)	45.52 ± 1.76	72.06 ± 1.64[c]	69.08 ± 0.76[a]	68.38 ± 1.14[b]	54.56 ± 1.49[c]	47.27 ± 1.52[c]
Brain TBARS (nM /mg protein)	11.67 ± 1.63	43.00 ± 2.96[c]	38.33 ± 1.21[a]	37.33 ± 2.33[b]	25.17 ± 3.54[c]	15.50 ± 1.51[c]
Brain SOD (U/min/mg protein)	1.88 ± 0.06	0.81 ± 0.06[c]	0.96 ± 0.05[a]	1.15 ± 0.10[a]	1.51 ± 0.06[c]	1.76 ± 0.08[c]
Brain GSH (μM/mg protein)	9.46 ± 0.82	4.22 ± 0.40[c]	4.46 ± 0.36	5.60 ± 0.34[b]	7.26 ± 0.59[c]	8.67 ± 0.52[c]
Serum nitrite (μM/L)	13.78 ± 0.45	4.25 ± 0.53[c]	5.25 ± 0.31[a]	6.10 ± 0.536[c]	10.37 ± 0.86[c]	12.89 ± 0.49[c]
Serum homocysteine levels (μM/L)	4.56 ± 0.25	21.71 ± 0.68[c]	20.53 ± 1.15[a]	20.03 ± 0.53[b]	13.39 ± 0.48[c]	9.04 ± 0.30[c]

Data were analyzed using one-way ANOVA, followed by Tukey's multiple range tests. All the results are expressed as mean ± SEM of $n = 6$ animals. Significance was defined as follows: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; comparison of treated groups, control groups and negative control groups

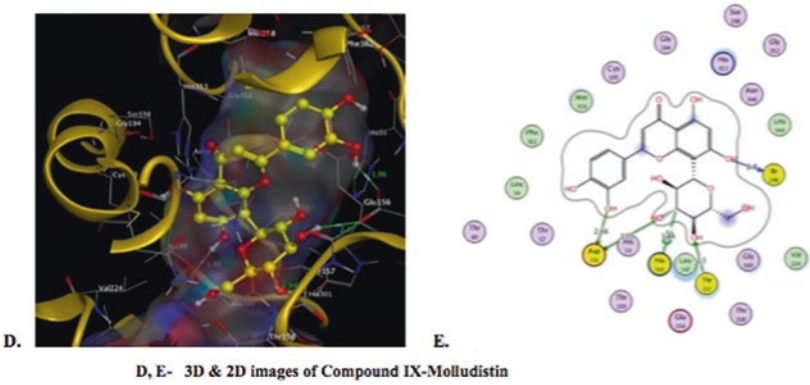
Table 3 The binding free energy dG (kcal/mol), ligand-protein interactions with distance, and energy of crystal ligand and *Ocimum sanctum* leaf chemical constituents

S. no	Compound	Type of bond, energy, distance, and interacting amino acid residues			Binding score
					dG
		H-donor	Distance	Energy	(kcal/mol)
1	Crystal ligand (neplanocin)	ASP-131	2.71	−2.4	−8.68
		ASP-131	2.93	−2.4	
		ASP-190	2.66	−3.5	
		GLU-59	2.94	−2.7	
		HIS-353	3.26	−1.2	
2	Molludistin (IX)	ASP-131	3.03	−2.4	−8.98
		ASP-131	2.66	−2.6	
		HIS-301	3.44	−0.6	
		ILE-299	2.80	−1.5	
		Thr-157	2.50	−0.5	
3	Apigenin (I)	ASN-346	3.16	−1.9	−6.55
		LYS-186	2.71	−7.4	
4	Ascorbic acid (II)	ASN-346	2.86	−2.3	−4.04
		LYS-186	3.88	−0.8	
5	β-Carotene (III)	–	–	–	−7.08
6	β-Caryophyllene (IV)	HIS-353	3.77	−1.1	−4.36
7	Carvacrol (V)	LEU-347	4.02	−0.8	−3.96
8	Eugenol (VI)	LEU-347	4.00	−0.7	−3.47
9	Luteolin (VII)	ASN-346	3.03	−2.5	−6.62
		HIS-55	3.10	−0.9	
		LYS-186	2.66	−9.2	
10	Methyl eugenol (VIII)	ASP-190	3.12	−0.8	2.89
11	Orientin (X)	ASP-190	2.55	−0.8	−5.53
		MET-358	3.37	−1.3	
		GLU-156	2.34	6.1	
		LYS-186	3.08	−4.9	
		LEU-347	4.06	−0.7	





B, C- 3D & 2D images of Crystal Ligand-Neplanocin



D, E- 3D & 2D images of Compound IX-Molludistin

Fig. 4 (**a**) *Ocimum sanctum* leaf chemical active constituents. (**b–e**) 2D and 3D docked structure of IX and crystal ligand (ball and stick) at SAH active site; hydrogen bond (green)

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Conflict of Interest The authors declare no conflict of interest.

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