ACS Chemical Neuroscience

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ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/acschemneuro.6b00313 • Publication Date (Web): 24 Oct 2016

ACS Publications

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Investigations on 16-arylideno steroids as a new class of neuroprotective agents for the treatment of Alzheimer's and Parkinson's diseases

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ABSTRACT: Neuroinflammatory mechanisms mediated by activated glial and cytokines (TNF-α, IL-1β) might contribute neuronal degeneration leading to Alzheimer's (AD) and Parkinson's disease (PD). Lipopolysaccharide (LPS) is an inflammogen derived from the cell wall of Gram-negative bacteria, which promotes neuroinflammation and subsequent neurodegeneration. Dehydroepiandrosterone (DHEA) and testosterone have been reported as neuroprotective steroids useful for the treatment of various neurodegenerative disorders. In the present study, several 16-arylidene steroidal derivatives have been evaluated as neuroprotective agents in LPS-treated animal models. It was observed that 16-arylidene steroidal derivatives **1a-d** and **6a-h** considerably improve LPS-induced learning, memory and movement deficits in animal models. Biochemical estimations of brain serum of treated animals revealed suppression of oxidative and nitrosative stress, acetylcholinesterase activity and reduction in TNF-α levels, which were induced through LPS mediated neuroinflammatory mechanism leading to neurodegeneration of brain. Of all the steroidal derivatives, 16-(4-pyridylidene) steroid **1c** and its 4-aza analogue **6c** were found to be the most active neuroprotective agents and produced effects comparable to standard drug celecoxib at a much lower dose and better than dexamethasone at the same dose in terms of behavioural, biochemical and molecular aspects.

Key words: Parkinson's disease, Alzheimer's disease, neuroinflammation, 16-arylidene steroids

INTRODUCTION

Central nervous system (CNS) in response to infections, trauma, stroke, toxins and other stimuli activates neuroinflammatory reaction within the brain.[1] Chronic neuroinflammation is usually associated with several neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD).[2,3] The amyloid-β(Aβ) plaques and neurofibrillary tangles are the main culprits for the pathology of Alzheimer's Disease (AD), which is characterized by loss of memory and learning abilities. The neuroinflammatory process in AD leads to activation of brain cells like microglia and astrocytes and also cytokines, chemokines and the complement system, which result in neuronal dysfunction and brain cell death. The over expression of cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 enhances amyloid precursor protein (APP) production and the amyloidogenic processing of APP that lead to the formation of amyloid β-42 (Aβ-42) peptide and hyperphosphorylation of tau (τ) protein.[4] Chronic neuroinflammation in PD causes activated glial and peripheral immune cells mediated degeneration of dopaminergic neurons leading to motor dysfunction.[5] In the striatum of patients with PD, increased concentration of key mediators of neuroinflammation like TNF-α, β2 microglobulin, interleukin 1β, 6 and 2 has been found. This further results in oxidative stress and cytokine-receptor-mediated apoptosis, which might eventually lead to neurodegeneration *via* dopaminergic cell death and hence disease progression.[6]

Lipopolysaccharide (LPS), a potent bacterial endotoxin elicits multiple pathological effects in human beings. In CNS, the exact mechanism of action of LPS remains unknown but it might be acting through the release of cytokines. Recent evidence shows that chronic infusion of LPS cause neurodegeneration resulting in impaired spatial memory and locomotor.[2,3,5] In addition, the literature reports reveal binding of LPS to specific transmembrane receptors such as CD14, LPS-binding protein (LBP)

and the toll-like receptor (TLR) in order to release cytokines (IL-1β, TNF-α and IL-6), NO, O_2^- and other inflammatory mediators. This leads to neuronal damage or death through nitrated/oxidative stress, mitochondrial dysfunction and apoptosis.[7]

During the last decades, steroids substituted with heterocycles at A- or D- ring of the steroid skeleton have garnered much attention due to their widespread pharmacological activities such as anti-inflammatory, anticancer and antimicrobial.[8-11] DHEA analogues with C3- or C17- modifications have displayed neuroprotective activity against the neural-crest derived PC12 cell model of serum deprivation-induced apoptosis.[12,13] 17β-*O*-Alkyl ethers of estradiol exhibited increased protection of neural (HT-22) cells against oxidative stress as compared to estradiol.[14] Testosterone prevent N-methyl-D-aspartate (NMDA) excitotoxicity and amyloid induced neurotoxicity that are mainly involved in pathophysiology of neurodegenerative disorders like PD and AD.[15,16]

16-Substituted steroids represent a novel class of heterosteroids possessing diversified biological properties.[17-19] As these compounds are derivatives of DHEA and testosterone, which are established neuroprotective agents, we became interested in studying the neuroprotective effects of 16-arylidene steroids and their 4-aza analogues for the treatment of neurodegenerative disorders like AD, PD and multiple sclerosis. Potent activity of 16-arylidene steroids during random screening of steroidal derivatives as neuroprotective agents further motivated us to pursue research work in this direction.

Literature further reveals the significance of pyridyl moiety for its protective action against the neurological disorders effecting behaviour and cognition.[20] The novel 3-pyridyl ethers have therapeutic potential as cholinergic channel modulators and can be useful for the treatment of cognitive impairments in patients with AD. The pyridyl moiety also acts as novel analogue of choline which promotes the formation and transmission of acetylcholine resulting in enhanced cognition and memory in patients suffering from neurodegenerative

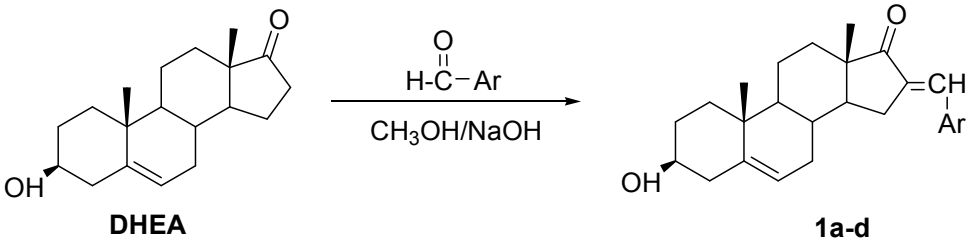
disorders.[21] Therefore in our present work a series of 16-pyridylidene steroids and their 4-aza analogues have been studied as neuroprotective agents using LPS-induced animal models of neuroinflammation.

RESULTS AND DISCUSSION

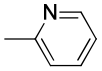
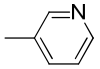
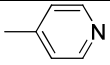
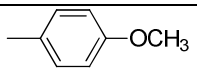
Chemical Synthesis

The synthesis of 16-arylidene steroids **1a-d** and 4-aza-16-arylidene steroids **6a-h** has been carried out as shown in schemes 1 and 2. Steroids **1a-d** were synthesized using base catalyzed Aldol Condensation of dehydroepiandrosterone with requisite aromatic aldehyde as reported earlier.[22,23](Scheme 1)

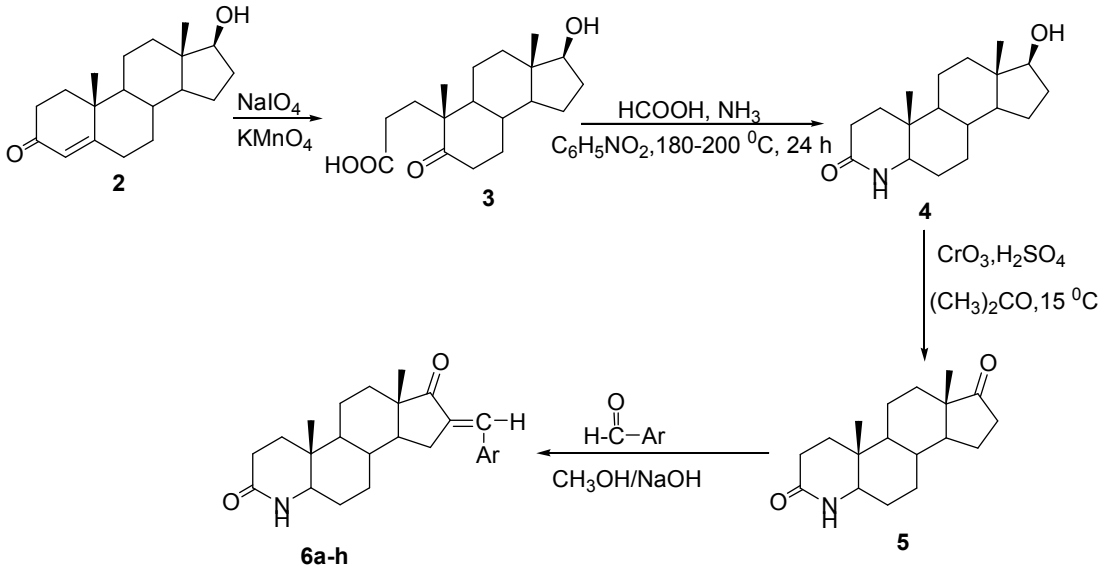
Results of initial studies indicated significantly better neuroprotective effects with 4-aza-16-arylidene analogues in comparison to their deaza counterparts, therefore 4-azasteroidal compounds were extensively explored by introducing diversely substituted arylidene moiety at 16-position of steroid skeleton. For the synthesis of 4-aza-16-arylidene steroids **6a-h**, testosterone (**2)** was oxidized using permanganate/periodate solution in *tert*-butanol to obtain an intermediate seco-keto acid **3**, which was further subjected to the Leukart reaction to afford 4-azasteroid **4**.[24] Subsequent Jone's oxidation gave 4-aza-androstan-3,17-dione **(5)**, which when further subjected to base catalyzed cross Aldol condensation using various aromatic aldehydes afforded corresponding 4-aza-16-arylidene derivatives **6a-h** as depicted in scheme 2.



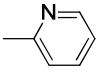
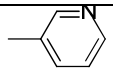
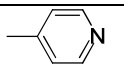
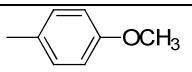
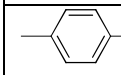
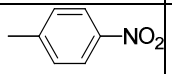
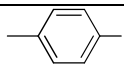
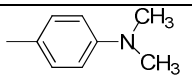
DHEA 1a-d

	a	b	c	d
Ar				

Scheme 1. Synthesis of 16-arylidene steroids **1a-d**



2 3 4 5 6a-h

	a	b	c	d
Ar				
	e	**f**	**g**	**h**
				

Scheme 2. Synthesis of 4-aza-16-arylidene steroids **6a-h**

Biological Activity

Neuroinflammation is now recognised as an important pathophysiological feature of various neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, however the exact relationship and mechanism is still unclear. In the current study, we have made an effort to establish a relationship between LPS induced neuroinflammation and consequent neurodegeneration leading to impairment of learning, memory and locomotion. It has been reported recently that systemic LPS injection promotes amyloidogenesis through activation of β and γ secretase, which cause rise in Aβ 1–42 levels in the cortex and hippocampus. Aβ oligomers are the principal toxins leading to neurite breakage and neuronal death. LPS induced Aβ 1–42 generation in both cortex and hippocampus plays a central role in the pathogenesis of Alzheimer's disease characterized by memory and learning deficits.[4] Literature also indicates that sub-toxic dose levels of LPS in substantia nigra (SN) induce neurodegeneration through neuronal loss, microglial activation, and pro-inflammatory phenotype with increased production of cytokines that would cause destruction of dopaminergic markers resulting in impairment of locomotor activity.[25]

Neurodegenerative processes initiated with activation of microglial cells may contribute in the release of pro-inflammatory and cytotoxic factors mainly TNF-α, IL-1β and finally resulting in memory impairment and motor dysfunction. The anti-inflammatory drugs could be useful in the treatment of neuroinflammation and could help in the prevention of neurodegeneration induced through so many pro-inflammatory mediators such as TNF-α, IL-1β, and COX-2. Non steroidal anti-inflammatory drugs such as ibuprofen and celecoxib reduce Aβ levels and brain inflammation in Tg2576 AD mice possibly through the inhibition of COX enzyme and could reduce the risk of AD.[26] Since a long time glucocorticoids are mainly used as potent anti-inflammatory drugs against brain inflammation and spinal cord injury in clinical neurology. The recent reports prove that dexamethasone produce protective

effects against neuroinflammation and also block the activation of microglia as well as inflammatory cytokines (TNF-α, IL-1β and IFN-γ) and is useful against dopaminergic degeneration in PD.[25]

DHEA and testosterone are established neurosteroids useful against various neurodegenerative disorders such as AD, PD and multiple sclerosis. DHEA and DHEAS protect the neurons against oxidative stress and also promote the neurogenesis in CNS. Mainly DHEA plays a key role to reduce inflammation in brain providing neuroprotection with improved cognitive function and memory enhancement. DHEA also potentiate locomotor activity of hemi-Parkinsonian monkey in MPTP-treated animals model.[27] Testosterone received an attractive consideration in the arena by researchers for improvement in motor and cognition functions in PD and AD, respectively. Testosterone prevents NMDA excitotoxicity and amyloid induced neurotoxicity. Recent studies have proven that testosterone supplementation in aged rats decrease the glial fibrilary acidic protein (GFAP) involved in neurodegenerative disorders through inflammatory cytokine release and reactive oxidative stress. Testosterone related up regulation of nerve growth factor (NGF) and its receptors play a promising role against inflammatory mediators released after brain injury.[15,28]

In light of these observations, we screened neuroprotective effects of the DHEA and testosterone derived 16-arylideno steroids **1a-d** and **6a-h** in LPS induced memory impairment and locomotor dysfunction. In the present study the administration of LPS in mice (250 µg/kg i.p.) and rats (10 µg in 2µl of 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M; pH 7.4), intranigrally) resulted in neuroinflammation. Behavioural paradigms for neurodegeneration including learning, memory and movement were assessed using Morris water maze, elevated plus maze, photoactometer and block test. Celecoxib and dexamethasone were used as standard compounds.

Behavioural studies on mice injected with LPS intraperitoneally.

The neuroprotective effects of 16-arylidene steroids **1a-d** and **6a-h** (5 mg/kg, i.p.) and standard compounds celecoxib (20 mg/kg, i.p.) and dexamethasone (5 mg/kg, i.p.) on LPS induced memory impairment and learning disability in mice were studied using Morris water maze and elevated plus maze models.[29] The results are compared with only LPS treated and control group. The values are shown as mean ± S.E.M and results are expressed as escape latency and TSTQ (time spent in target quadrant) in Morris water maze and % ITL (initial transfer latency) in elevated plus maze models (Table 1).

Table 1. Neuroprotective effects of 16-arylidene steroids on mice injected with LPS intraperitoneally

	Morris Water Maze		Elevated Plus Maze
Compound	Escape Latency (s)	TSTQ (s)	% ITL (s)
Control	41.5 ± 1.9	55 ± 3.4	11.4 ± 2.6
LPS	73.2 ± 1.6*	13 ± 2.9*	91.4 ± 2.9*
Celecoxib	52.9 ± 2.4$^{\#}$	44 ± 3$^{\#}$	24.4 ± 2.2$^{\#}$
Dexamethasone	56.7 ± 2.5$^{\#}$	40 ± 3$^{\#}$	31.1 ± 2.4$^{\#}$
1a	62.5 ± 1.8$^{\#}$	35 ± 4$^{\#}$	37.7± 2.2$^{\#}$
1b	65.2 ± 2$^{\#}$	32 ± 3$^{\#}$	40 ± 2.7$^{\#}$
1c	57.9 ± 2$^{\#}$	40 ± 4$^{\#}$	30.2 ± 4.4$^{\#}$
1d	60.3 ± 2.3$^{\#}$	38 ± 3$^{\#}$	33.8 ± 2.8$^{\#}$
6a	61.6 ± 1.4$^{\#}$	37 ± 2$^{\#}$	35.1 ± 3.3$^{\#}$
6b	63.5 ± 1.9$^{\#}$	38 ± 2$^{\#}$	33.3 ± 3.3$^{\#}$
6c	55.7 ± 2.0$^{\#}$	42 ± 4$^{\#}$	28.8 ± 2.2$^{\#}$
6d	65.1 ± 1.4$^{\#}$	36 ± 4$^{\#}$	35.5 ± 2.2$^{\#}$
6e	62.8 ± 1.5$^{\#}$	38 ± 4$^{\#}$	33.8 ± 1.6$^{\#}$
6f	59.7 ± 2.3$^{\#}$	39 ± 3$^{\#}$	32 ± 3.3$^{\#}$
6g	66.0 ± 1.4$^{\#}$	37 ± 4$^{\#}$	34.4 ± 2.2$^{\#}$
6h	66.6 ± 1.6$^{\#}$	36 ± 3$^{\#}$	35.5 ± 2.2$^{\#}$

*$p<0.05$ as compared to control; #$p<0.05$ as compared to LPS (n=5). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$).

The behavioural assessment for memory impairment in mice treated with LPS intraperitoneally depicted an increase in escape latency in Morris water maze task (from day 1 to day 5) and % ITL in elevated plus maze due to the LPS induced dysfunction in learning and memory. Treatment with synthesized derivatives **1a-d** and **6a-h** (5mg/kg) resulted in

significantly reduced escape latency and % ITL. In probe trial, significantly decreased TSTQ in LPS treated mice was appreciably reversed on treatment with 16-arylideno steroids **1a-d** and **6a-h**. The data obtained from elevated plus maze model further substantiated the findings of Morris water maze test as treatment with steroidal derivatives considerably reduced the otherwise increased % ITL, after 24 h of training period in mice. The 4-pyridylidene steroids **1c** and **6c** emerged as the most potent compounds and significantly improved the learning and memory functions at 5 mg/kg, which are comparable with standard drugs celecoxib (20 mg/kg) and better than dexamethasone (5 mg/kg). The effects of steroids **1c** and **6c** on the behavioural pattern in mice treated with LPS intraperitoneally have been represented in Figure 1.

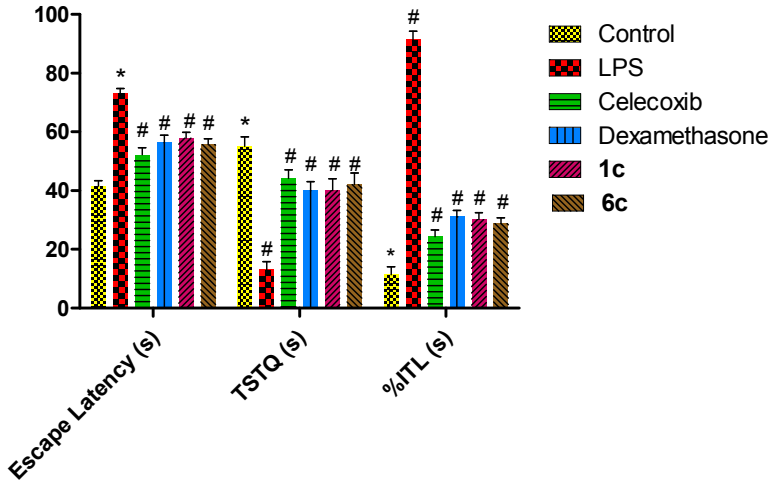


Figure 1. Behavioural observations for memory and learning deficits in mice treated with LPS intraperitoneally for compounds **1c** and **6c.** *$p<0.05$ as compared to control; #$p<0.05$ as compared to LPS (n=5). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and a value of $p<0.001$ was considered statistically significant.

Behavioural studies on rats injected with LPS intranigrally

The effects of 16-arylidene steroids **1a-d** and **6a-h** (2 mg/kg, i.p.) and standard drugs celecoxib (20 mg/kg, i.p.) and dexamethasone (2 mg/kg, i.p.) on behavioural pattern of intranigrally LPS-treated rats with respect to locomotor activity and cognition were studied using actophotometer, elevated plus maze and block test.[25,30] The results are compared with

sham, LPS and vehicle control group. The values are shown as mean ± S.E.M and results are expressed as ambulation and rearing in locomotor activity, % ITL in elevated plus maze and degree of catatonia using block test (Table 2).

Table 2. Neuroprotective effects of 16-arylidene steroids in rats injected with LPS intranigrally

	Locomotor Activity		Elevated Plus Maze	Catatonic Response
Compound	Ambulation (min)	Rearing (min)	% ITL (s)	Degree of Catatonia (score)
Sham	300 ± 2.5	145 ± 2.0	16.6 ± 3.2	0
LPS + Vehicle	56.3 ± 3.8*	16.3 ± 3.3*	83.3 ± 4.4*	1.2 ± 0.02*
LPS	55.8 ± 4.6$^{\#}$	16.0 ± 2.3$^{\#}$	82.9 ± 3.1$^{\#}$	1.22 ± 0.03$^{\#}$
Celecoxib	215 ± 3.8$^{\$}$	73.3 ± 2.0$^{\$}$	31.3 ± 2.0$^{\$}$	0.21 ± 0.04$^{\$}$
Dexamethasone	200± 3.9$^{\$}$	63± 3.0$^{\$}$	37.1± 2.2$^{\$}$	0.33 ± 0.05$^{\$}$
1a	181.5 ± 2.9$^{\$}$	53± 3.2$^{\$}$	48.3± 3.0$^{\$}$	0.52 ± 0.03$^{\$}$
1b	164 ± 4$^{\$}$	47 ± 4$^{\$}$	51.6 ± 4$^{\$}$	0.57 ± 0.06$^{\$}$
1c	194.5 ± 4.4$^{\$}$	63 ± 3.4$^{\$}$	39.7 ± 3.0$^{\$}$	0.45 ± 0.05$^{\$}$
1d	190 ± 3.5$^{\$}$	57 ± 2.8$^{\$}$	44.6 ± 3.3$^{\$}$	0.48 ± 0.04$^{\$}$
6a	192 ± 3.0$^{\$}$	49.3 ± 4.2$^{\$}$	46 ± 2$^{\$}$	0.49 ± 0.04$^{\$}$
6b	189 ± 2.6$^{\$}$	56 ± 2.7$^{\$}$	48.9 ± 2.9$^{\$}$	0.52 ± 0.06$^{\$}$
6c	202.3 ± 4.3$^{\$}$	67 ± 3.6$^{\$}$	37.1 ± 3.3$^{\$}$	0.42 ± 0.04$^{\$}$
6d	187 ± 4.3$^{\$}$	55 ± 3.4$^{\$}$	52 ± 2.9$^{\$}$	0.50 ± 0.04$^{\$}$
6e	193.3 ± 3.9$^{\$}$	57± 3.0$^{\$}$	47 ± 3.3$^{\$}$	0.49 ± 0.04$^{\$}$
6f	200.6 ± 4.1$^{\$}$	60 ± 2.8$^{\$}$	42.3 ± 3.0$^{\$}$	0.47 ± 0.03$^{\$}$
6g	183.3 ± 3.4$^{\$}$	53.6 ± 3.2$^{\$}$	51.6 ± 3.2$^{\$}$	0.52 ± 0.04$^{\$}$
6h	176.6 ± 3.5$^{\$}$	50.6 ± 3.2$^{\$}$	55.6 ± 3.4$^{\$}$	0.54 ± 0.06$^{\$}$

*$p<0.05$ as compared to sham; #$p<0.05$ as compared to LPS + vehicle; \$$p<0.05$ as compared to LPS (n=5). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$).

The behavioural assessment for memory and locomotor impairment in rats treated with LPS intranigrally indicated a significant decline in the locomotor activity in LPS treated and vehicle control group rats as compared to sham after 21 days. The catatonic score and % ITL also increased notably in LPS and control group analysed at 21st day of experimental protocol. The group treated with daily i.p. injections of steroidal derivatives **1a-d** and **6a-h** (2 mg/kg) for 21 days showed improvement in the locomotor activity with significant decrease in degree of catatonia and % ITL as compared to LPS and LPS + vehicle group in the

designed period of experimental protocol. The significant improvement in locomotion, learning ability and catatonia after treatment with compounds **1c** and **6c** is found to be better than dexamethasone (2 mg/kg) and comparable to celecoxib (20 mg/kg) as shown in Figures 2 and 3. The improvement in locomotion, catatonia and learning abilities on treatment with 4-aza-16-arylidiene **6c** was found to be more than 4-deaza-16-arylidiene steroid **1c**.

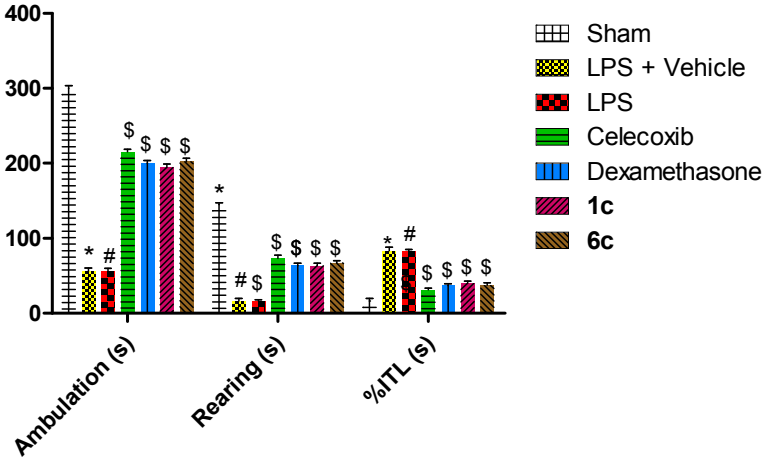


Figure 2. Behavioural changes with compounds **1c** and **6c** for locomotion and memory deficits in rats treated with LPS intranigrally. * $p<0.05$ as compared to sham; #$p<0.05$ as compared to LPS + vehicle; \$$p<0.05$ as compared to LPS (n=5). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$)

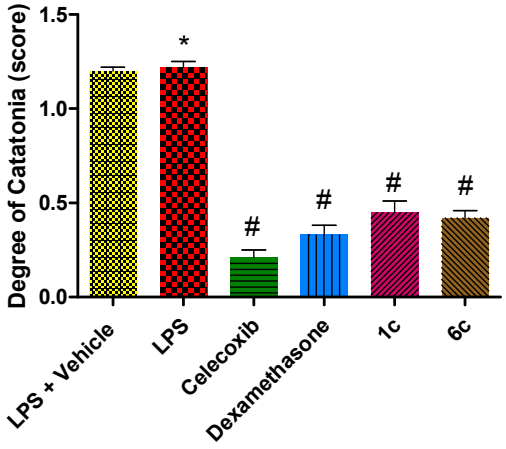


Figure 3. Behavioural changes with compounds **1c** and **6c** with respect to catatonic response in rats treated with LPS intranigrally *$p<0.05$ as compare to LPS + vehicle; #$p<0.05$ as compare to LPS (n=5). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$)

Biochemical estimations in brain supernatants of mice and rats

Acetylcholine is a major parasympathetic neurotransmitter that inhibits LPS-induced production of proinflammatory cytokines such as IL-1, TNF-α from macrophages and activated microglia. The neurotransmitter acetylcholine is degraded by the enzyme acetylcholinesterase leading to memory impairment. The significant increase in the AChE activity by LPS treatment results in decreased cholinergic activity. In addition, an up-regulation of proinflammatory cytokines (TNF-α and IL-1β) on injecting LPS mainly cause neurotoxic effects leading to neuron cell death.[31] LPS is also suggested to increase iNOS activation and thereby nitric oxide (NO) production. NO can damage DNA and irreversibly modifies proteins such as tyrosine nitration and thiol oxidation, which are common pathogenic mechanisms in several neurodegenerative diseases. LPS administration is also associated with increased generation of reactive oxygen species (ROS) from highly metabolically active mitochondria which further stimulate toll like receptor signalling components to propagate. These ROS such as superoxide anion, hydrogen peroxide and nitric oxide exert harmful effects on brain homeostasis. Recent studies have revealed that excessive production and accumulation of NO after LPS administration lead to neurodegeneration. ROS released from activated microglia or astrocytes may interact with lipid components of cellular membrane, initiating lipid peroxidation that results in the breakdown of the lipid constituents into highly reactive by-products.[32] Under LPS induced oxidative stress the mismatch between the production of free radicals and the ability to scavenge them take place. Intracerebroventricular administration of LPS causes significant decrease in the activity of antioxidants such as reduced glutathione, superoxide dismutase and catalase in cerebral cortex and hippocampus regions of brain.[31]

In the current study, the biochemical parameters acetylcholinesterase, lipid peroxidation, nitrite and oxidative stress were estimated in the brain supernatant of mice

(i.p. LPS) and rats (i.g. LPS) treated with 16-arylidene steroids **1a-d** and **6a-h** along with standard drugs celecoxib and dexamethasone as reported earlier.[33] Results are expressed as μmoles of AChE/min/mg protein in AChE Assay, n moles of MDA/mg protein in LPO Assay, Nitrite concentration (μg/ml) in Nitrite Assay, n moles of GSH/mg protein in GSH Assay, SOD units/mg protein in SOD Assay and μmoles of H_2O_2 decomposed/min/mg protein in Catalase Assay. The values are shown as mean ± S.E.M for mice and rat brain supernatants in Tables 3 and 4, respectively.

A significant increase in AchE, malondialdehyde and nitrite levels along with marked reduction in reduced glutathione and enzymatic activity of superoxide dismutase and catalase was observed in the brain supernatants of LPS treated mice and rats. Of all the compounds, the treatment with 16-(4-pyridylidene)steroid **1c** and its 4-aza analogue **6c** helped to restore maximum normalcy of the biochemical mediators along with marked inhibition of reactive oxygen and nitrogen species involved in mechanism of neuroinflammation. The biochemical estimation of the acetylcholinesterase, lipid peroxidation, nitrite and oxidative stress in mice and rat brain homogenates on treatment with most active compounds **1c** and **6c** have been illustrated in Figures 4 and 5.

Inhibition of TNF-α

TNF-α plays an important role in progression of neuroinflammation as is observed from the various studies reported in literature. Therefore the estimation of TNF-α activity has been carried out in the brain serum of rats treated with 16-arylidene steroids **1a-d** and **6a-h** and compared with standards celecoxib and dexamethasone using TNF-α immunoassay kit.[34] The inhibition of TNF-α is expressed in pg/mg protein and values are shown as mean ± S.E.M (Table 5).

Table 3. Estimation of biochemical parameters in brain supernatant of mice treated with LPS intraperitonially

	AchE Assay	LPO Assay	Nitrite Assay	GSH Assay	SOD Assay	Catalase Assay
Compound	µmoles of AchE/min/mg protein	n moles of MDA/mg protein	Nitrite conc.(µg/ml)	µmoles of GSH/mg protein	SOD units/ mg protein	Catalase activity/min
Control	0.0015 ± 0.0003	1.9 ± 0.1	287.5 ± 35	0.0078 ± 0.0003	6.5 ± 1.8	4.9 ± 0.3
LPS	0.0051 ± 0.0003*	7.5 ± 0.3*	730.1 ± 28*	0.0034 ± 0.0005*	1.5 ± 0.7*	1.1 ± 0.4*
Celecoxib	0.0026 ± 0.0003#	3.0 ± 0.1#	434.4 ± 31.1#	0.0061 ± 0.0005#	4.9 ± 1.6#	3.6 ± 0.3#
Dexamethasone	0.0030 ± 0.0004#	3.5 ± 0.1#	467.1 ± 32.5#	0.0056 ± 0.0003#	4.3 ± 1.3#	3.2 ± 0.6#
1a	0.0036 ± 0.0005#	3.9 ± 0.2#	537.8 ± 29.8#	0.0048 ± 0.0004#	3.4 ± 1.1#	2.8 ± 0.8#
1b	0.0039 ± 0.0004#	4.3 ± 0.2#	563.5 ± 28.9#	0.0044 ± 0.0002#	3.0 ± 1.2#	2.5 ± 0.6#
1c	0.0031 ± 0.0003#	3.5 ± 0.2#	503.6 ± 30.5#	0.0052 ± 0.0004#	3.9 ± 1.4#	3.1 ± 0.5#
1d	0.0034 ± 0.0004#	3.7 ± 0.2#	513.4 ± 28.4#	0.0050 ± 0.0005#	3.7 ± 1.6#	3.0 ± 0.4#
6a	0.0034 ± 0.0006#	3.8 ± 0.1#	525.8 ± 32.8#	0.0050 ± 0.0006#	3.7 ± 1.5#	2.9 ± 0.6#
6b	0.0036 ± 0.0004#	4.0 ± 0.2#	548.2 ± 29.5#	0.0049 ± 0.0005#	3.5 ± 1.6#	2.7 ± 0.4#
6c	0.0028 ± 0.0004#	3.3 ± 0.2#	485.8 ± 33.8#	0.0054 ± 0.0004#	4.1 ± 1.4#	3.2 ± 0.7#
6d	0.0033 ± 0.0004#	3.6 ± 0.2#	510.4 ± 32.7#	0.0051 ± 0.0003#	3.8 ± 1.3#	3.0 ± 0.4#
6e	0.0035 ± 0.0006#	3.9 ± 0.2#	539.7 ± 35#	0.0047 ± 0.0003#	3.4 ± 1.8#	2.9 ± 0.4#
6f	0.0035 ± 0.0003#	3.8 ± 0.2#	531.2 ± 30.5#	0.0049 ± 0.0003#	3.5 ± 1.1#	2.9 ± 0.6#
6g	0.0034 ± 0.0004#	3.9 ± 0.1#	535.2 ± 31.3#	0.0047 ± 0.0003#	3.5 ± 1.6#	2.8 ± 0.7#
6h	0.0035 ± 0.0003#	4.1 ± 0.1#	544.5 ± 34#	0.0045 ± 0.0003#	3.3 ± 1.5#	2.7 ± 0.5#

Results are expressed as µmoles of AchE/min/mg protein in AchE Assay, n moles of MDA/mg protein in LPO Assay, Nitrite conc.(µg/ml) in Nitrite Assay, µmoles of GSH/mg protein in GSH Assay, SOD units/ mg protein in SOD Assay and Catalase activity/min in Catalase Assay . The values are shown as mean ± S.E.M. * $p<0.05$ as compared to control; #$p<0.05$ as compared to LPS (n=5 per group). One- way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values are considered statistically significant ($p<0.001$).

Table 4. Estimation of biochemical parameters in brain supernatants of rats treated with LPS intranigrally

	AChE Assay	LPO Assay	Nitrite Assay	GSH Assay	SOD Assay	Catalase Assay
Compound	µmoles of AChE/min/mg protein	n moles of MDA/mg protein	Nitrite conc.(µg/ml)	µmoles of GSH/mg protein	SOD units/ mg protein	Catalase activity/min
Sham	0.0032 ± 0.0002	3.2 ± 0.2	419.5 ± 26.1	0.048 ± 0.002	9.8 ± 1.4	7.0 ± 0.4
LPS + Vehicle	0.0096 ± 0.0005*	9.5 ± 0.4*	914.1 ± 25.2*	0.021 ± 0.001*	2.7 ± 1.0*	2.2 ± 0.4*
LPS	0.0097 ± 0.0003$^{\#}$	9.4 ± 0.6$^{\#}$	915.8 ± 25.4$^{\#}$	0.020 ± 0.001$^{\#}$	2.8 ± 1.1$^{\#}$	2.1 ± 0.3$^{\#}$
Celecoxib	0.0055 ± 0.0003$^{\$}$	5.4 ± 0.4$^{\$}$	523.1 ± 20.1$^{\$}$	0.036 ± 0.003$^{\$}$	6.6 ± 1.5$^{\$}$	5.5 ± 0.5$^{\$}$
Dexamethasone	0.0062 ± 0.0007$^{\$}$	6.0 ± 0.3$^{\$}$	565.8 ± 27.3$^{\$}$	0.033 ± 0.001$^{\$}$	6.2 ± 1.7$^{\$}$	5.1 ± 0.3$^{\$}$
1a	0.0068 ± 0.0003$^{\$}$	6.7 ± 0.5$^{\$}$	640.5 ± 22.8$^{\$}$	0.028 ± 0.003$^{\$}$	5.2 ± 1.3$^{\$}$	4.3 ± 0.5$^{\$}$
1b	0.0070 ± 0.0005$^{\$}$	7.0 ± 0.3$^{\$}$	673.8 ± 22.9$^{\$}$	0.026 ± 0.002$^{\$}$	4.9 ± 1.3$^{\$}$	4.0 ± 0.4$^{\$}$
1c	0.0063 ± 0.0003$^{\$}$	6.3 ± 0.4$^{\$}$	595.8 ± 24.9$^{\$}$	0.033 ± 0.002$^{\$}$	5.9 ± 1.9$^{\$}$	4.9 ± 0.3$^{\$}$
1d	0.0065 ± 0.0004$^{\$}$	6.5 ± 0.6$^{\$}$	619.4 ± 27.4$^{\$}$	0.030 ± 0.004$^{\$}$	5.6 ± 1.2$^{\$}$	4.6 ± 0.8$^{\$}$
6a	0.0066 ± 0.0007$^{\$}$	6.5 ± 0.6$^{\$}$	623.1 ± 26.8$^{\$}$	0.030 ± 0.003$^{\$}$	5.4 ± 1.0$^{\$}$	4.5 ± 0.6$^{\$}$
6b	0.0068 ± 0.0004$^{\$}$	6.8 ± 0.5$^{\$}$	649.3 ± 25.5$^{\$}$	0.028 ± 0.005$^{\$}$	5.2 ± 1.6$^{\$}$	4.3 ± 0.5$^{\$}$
6c	0.0059 ± 0.0003$^{\$}$	6.1 ± 0.7$^{\$}$	578.5 ± 23.8$^{\$}$	0.035 ± 0.002$^{\$}$	6.2 ± 1.5$^{\$}$	5.1 ± 0.6$^{\$}$
6d	0.0064 ± 0.0002$^{\$}$	6.4 ± 0.6$^{\$}$	604.5 ± 22.7$^{\$}$	0.031 ± 0.003$^{\$}$	5.7 ± 1.3$^{\$}$	4.7 ± 0.7$^{\$}$
6e	0.0068 ± 0.0005$^{\$}$	6.7 ± 0.5$^{\$}$	649.5 ± 20.5$^{\$}$	0.029 ± 0.002$^{\$}$	5.3 ± 1.8$^{\$}$	4.4 ± 0.3$^{\$}$
6f	0.0067 ± 0.0003$^{\$}$	6.6 ± 0.5$^{\$}$	640.1 ± 25.5$^{\$}$	0.030 ± 0.004$^{\$}$	5.5 ± 1.3$^{\$}$	4.5 ± 0.3$^{\$}$
6g	0.0068 ± 0.0005$^{\$}$	6.8 ± 0.6$^{\$}$	655.3 ± 25.3$^{\$}$	0.028 ± 0.004$^{\$}$	5.1 ± 1.4$^{\$}$	4.4 ± 0.3$^{\$}$
6h	0.0070 ± 0.0004$^{\$}$	6.9 ± 0.5$^{\$}$	664.1 ± 28.3$^{\$}$	0.027 ± 0.004$^{\$}$	5.0 ± 1.3$^{\$}$	4.2 ± 0.4$^{\$}$

Results are expressed as µmoles of AchE/min/mg protein in AchE Assay, n moles of MDA/mg protein in LPO Assay, Nitrite conc.(µg/ml) in Nitrite Assay, µmoles of GSH/mg protein in GSH Assay, SOD units/ mg protein in SOD Assay and Catalase activity/min in Catalase Assay . The values are shown as mean ± S.E.M. * $p<0.05$ as compared to sham; #$p<0.05$ as compared to LPS+ vehicle; \$$p<0.05$ as compared to LPS (n=5). One-way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$).

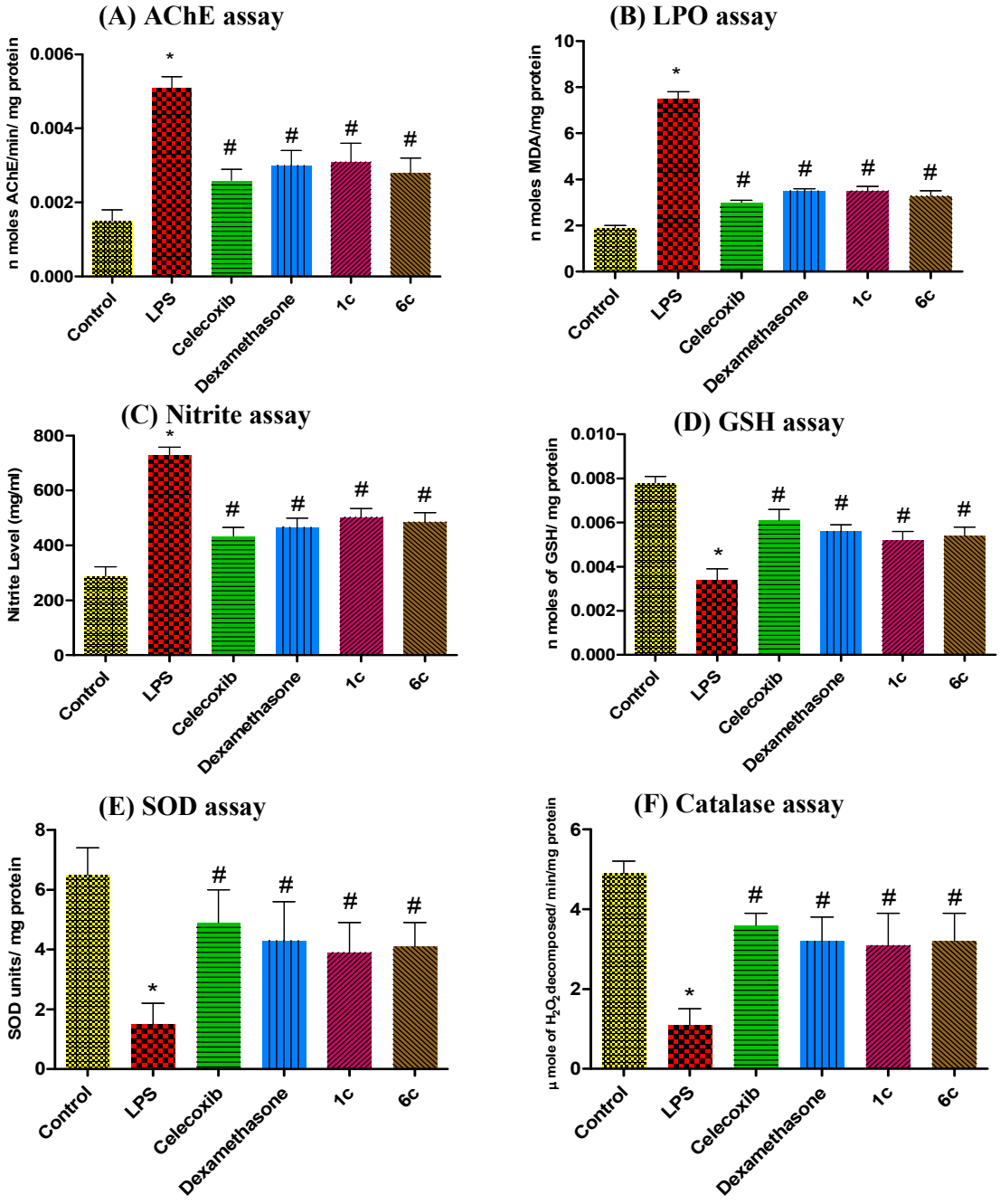


Figure 4. Biochemical estimations in brain supernatant of mice treated with compounds **1a** and **6c**. *p<0.05 as compared to control; #p<0.05 as compared to LPS (n=5). One-way ANOVA followed by Tukey's test was employed to find out the inter-group variations and values were considered statistically significant (p<0.001).

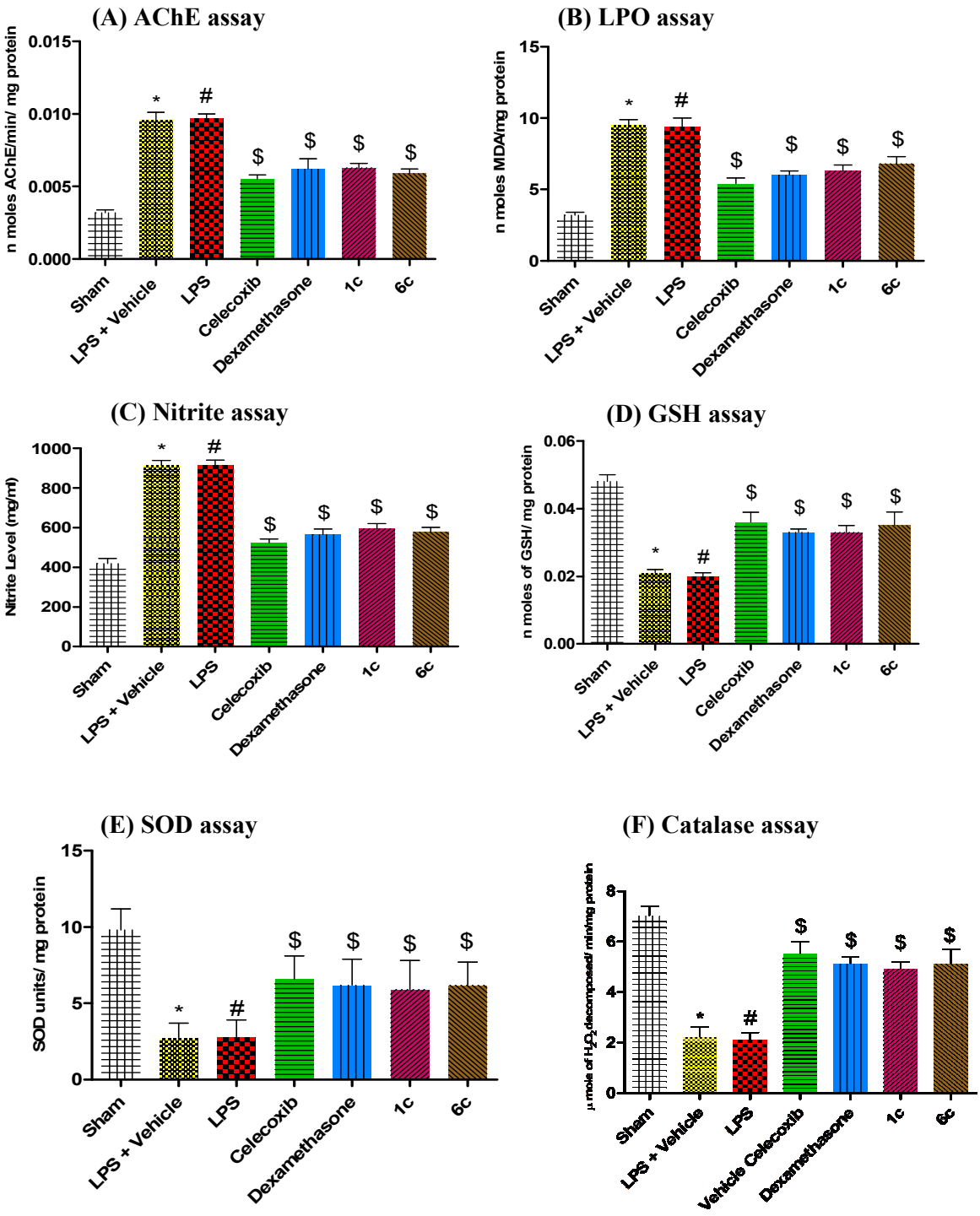


Figure 5. Biochemical estimation in brain supernatant of rats treated with compounds **1a** and **6c**. *p<0.05 as compared to sham; #p<0.05 as compared to LPS + vehicle; $p<0.05 as compared to LPS (n=5). One-way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant (p<0.001).

Table 5. Estimation of TNF-α levels in brain serum of rats

Compound	TNF-α (pg/mg protein)
Sham	48.4 ± 1.5
LPS + Vehicle	368.8 ± 1.9*
LPS	370.2 ± 1.6#
Celecoxib	68.2 ± 1.1$
Dexamethasone	89.6 ± 2.0$
1a	126.8 ± 3.0$
1b	139.2 ± 2.9$
1c	93.6 ± 1.4$
1d	107.2 ± 2.4$
6a	98.8 ± 3.1$
6b	131.2 ± 1.7$
6c	88.6 ± 1.8$
6d	104.8 ± 1.5$
6e	123.2 ± 2.2$
6f	100.6 ± 1.6$
6g	135.2 ± 1.9$
6h	114 ± 1.9$

* $p<0.05$ as compared to sham; #$p<0.05$ as compared to LPS + vehicle; \$$p<0.05$ as compared to LPS (n=5). One-way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant ($p<0.001$).

Interestingly, a significant elevation in the levels of TNF-α in the brain serum of rats treated with LPS + vehicle and LPS alone was observed. This indicates that enhancement of neuroinflammation results into neurodegeneration. LPS significantly increases the magnitude as well as duration of central and peripheral pro-inflammatory cytokines (TNF-α and IL-1β) suggesting a role of cytokines in LPS-induced neuroinflammation.[31] The treatment with 16-arylidene steroids **1a-d** and 4-aza-16-arylidene steroids **6a-h** resulted in decreased TNF-α levels in the brains of LPS injected rats. The 16-(4-pyridylidene)steroid **1c** and its 4-aza counterpart **6c** were found to be better than standard drugs in reducing TNF-α levels, the later being more active than the deaza analogue **1c** as shown in Figure 6.

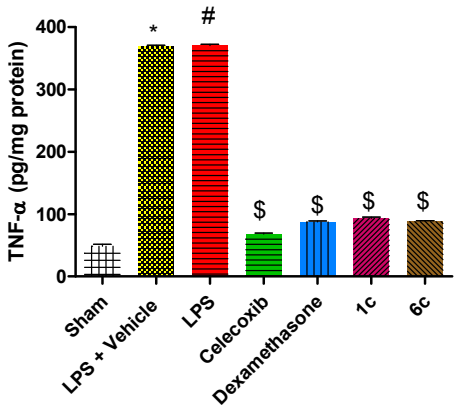


Figure 6. TNF-α levels in the brain serum of rats. *p<0.05 as compared to sham; #p<0.05 as compared to LPS + vehicle; $p<0.05 as compared to LPS (n=5). One-way ANOVA followed by Tukey's test was employed to find out the inter-group variation and values were considered statistically significant (p<0.001).

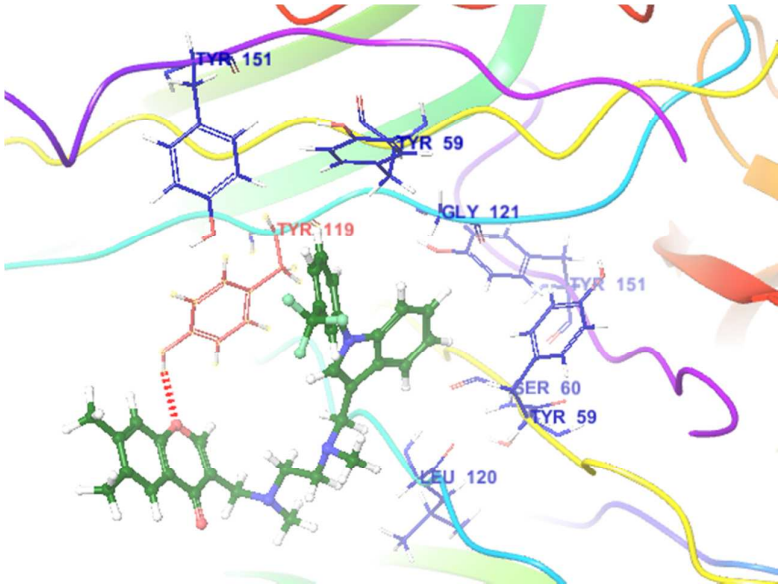
Docking Studies Analysis

TNF-α is one of the key elements involved in the molecular mechanism of neuroinflammation.[4-6] Observing the reduced TNF-α levels on treatment with the 16-arylidene steroids, it was thought worthwhile to study the three dimensional interactions of the compounds with the receptor using computational docking studies. The studies might be helpful in acquiring a tentative idea about one of the possible mechanism of the 16-arylidene steroidal derivatives as neuroprotective agents. Steroids **1a-d** and **6a-h** were docked against crystal structure of TNF-α and compared with reference compounds celecoxib and dexamethasone. The docking was done on the active site of 10 Å defined around center of co-crystallized ligand composed of trifluoromethylphenyl indole and dimethyl chromone moieties linked by a dimethylamine spacer. The docking protocol was validated by re-docking of the co-crystallized ligand back into the protein. The glide scores of the co-crystallized ligand, standard drugs (celecoxib and dexamethasone) and synthesized steroidal derivatives are given in Table 6. The 3D and 2D interactions of most active compounds **1c** and **6c** were compared with co-crystallized ligand and are represented in Figures 7 and 8, respectively.

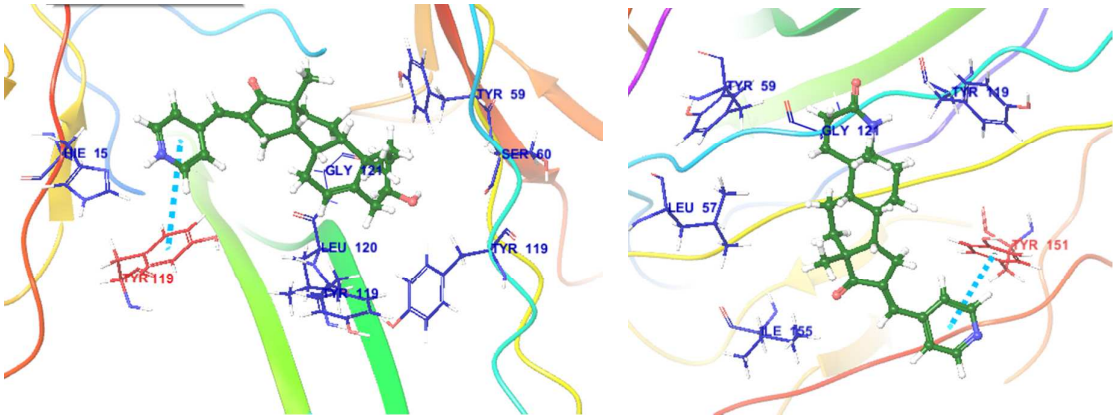
Table 6: Glide score of co-crystallized ligand, celecoxib, dexamethasone and steroidal derivatives on the active site of TNF-α.

Compound	Glide Score
Co-crystallized ligand	-4.302
Celecoxib	-4.616
Dexamethasone	-4.713
1a	-6.145
1b	-6.124
1c	-4.639
1d	-6.557
6a	-4.388
6b	-4.796
6c	-3.723
6d	-4.602
6e	-4.774
6f	-4.086
6g	-4.637
6h	-3.942

The selected TNF-α protein is a dimer consisting of 18 contact residues, of which ten are from chain A (Lys57, Lys58, Tyr59, Ser60, Gly61, Tyr119, Lys120, Gly121, Gly122, and Tyr151). The remaining eight are a subset of these residues from chain B (Lys57, Tyr59, Ser60, Gly61, Tyr119, Lys120, Tyr121 and Tyr151). The co-crystallized ligand consisting of trifluoromethylphenylindole and dimethyl chromone moieties folded back upon each other and the interactions were largely hydrophobic in nature. Co-crystallized ligand produced intra-molecular π–π stacking between the aromatic moieties. Six tyrosine residues (Tyr59, Tyr151 and Tyr119 residues from both chain A and chain B) provide solvent accessible surface for interacting with the ligand. Tyr119 was notable in being located close to the three fold symmetry axis of the TNF-α and in making contact with co-crystallized ligand.[35,36] Steroids **1c** and **6a** showed the similar interactions with tyrosine residues Tyr119 as co-crystallized ligand, which are essential for the inhibition TNF-α. 4-Aza-16-(4-pyridylidene) steroid **6c** showed the π-π stacking with the tyrosine residues Tyr151 responsible for the inhibition of TNF-α. 16-arylidene steroids **1d**, **6d, 6e** and **6h** also depicted the π-π stacking

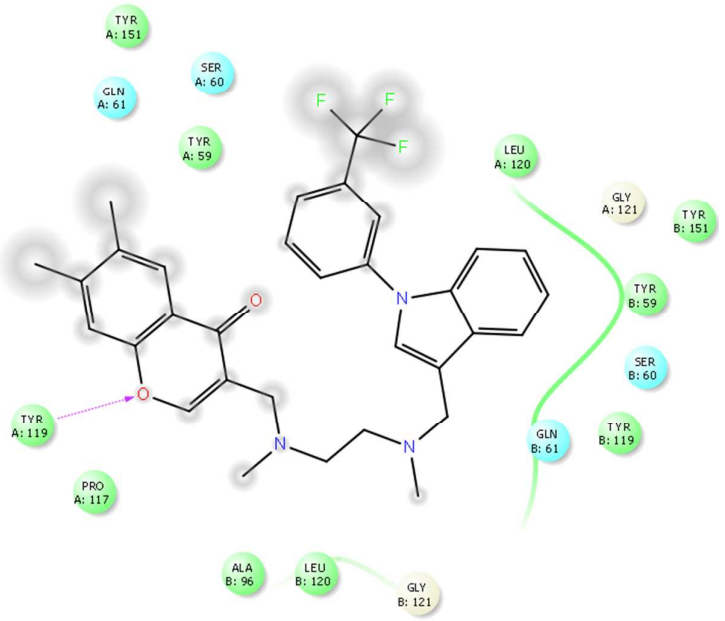


Co-crystallized ligand

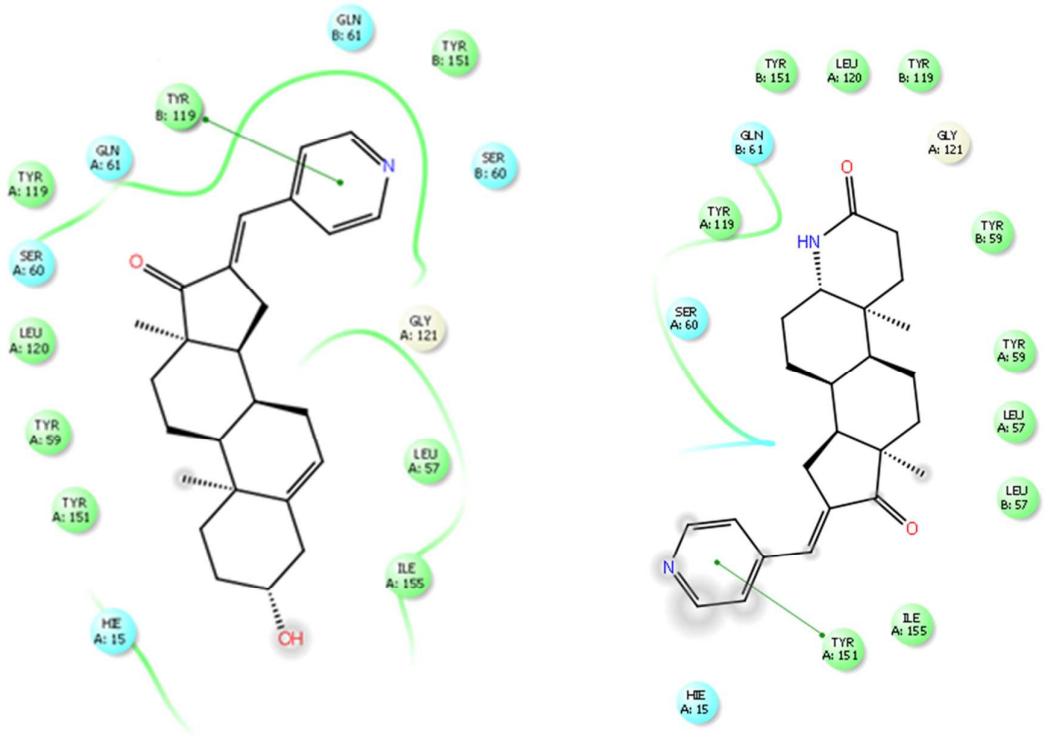


1c **6c**

Figure 7. The 3D interaction diagram of co-crystallized ligand, steroidal derivatives **1c** and **6c** at active site of TNF-α



Co-crystallized ligand



1c **6c**

Figure 8. The 2D interaction diagram of co-crystallized ligand, steroidal derivatives **1c** and **6c** at active site of TNF-α.

and hydrogen bonding interactions with tyrosine residues Tyr151 and Tyr 59, which are also required for the inhibition of TNF-α. Docking analysis further revealed that the solvent accessible surface interaction with tyrosine residues from both chain is important for TNF-inhibitory activity. Remaining compounds including standard (celecoxib and dexamethasone) were not able to bind with the active amino acids (Tyr59, Tyr151 and Tyr 119.) essential for TNF-α inhibition. The observed glide score of most active compounds **1c** and **6a-h** were comparable to that of parent co-crystallized ligand**.** It was observed that 4-azasteroids are more active against TNF-α, the main culprit of neuroinflammation. However, besides TNF-α there are many other factors such as interleukin 1β, 6 and 2, NO, O_2^- and several inflammatory mediators, which might be involved in the progression of neuroinflammation.[6]

Overall 16-arylidene steroids **1a-d** and **6a-h** significantly improved learning, memory and movement function and markedly suppressed LPS induced oxidative stress, nitrosative stress, acetylcholinesterase activity and enhanced TNF-α levels. In general, 4-azasteroids **6a-h** displayed superior potential as neuroprotective agents than their corresponding deaza analogues **1a-d**. Of all, introduction of 4-pyridylidene ring at 16-position of the steroid nucleus seems to be the most appropriate substitution for good neuroprotective activity as 16-(4-pyridylidene) steroid **1c** and its 4-aza analogue **6c** displayed highest potency and produced effects comparable to the standard drugs.

CONCLUSION

On the basis of behavioural, biochemical and molecular studies, it is concluded that that 16-arylidene steroids represent a new class of neuroprotective agents for the treatment of Alzheimer's and Parkinson's diseases. All the synthesized derivatives **1a-d** and **6a-h** prevent the progression of LPS-induced neuroinflammation and subsequent neurodegeneration possibly by inhibiting TNF-α or by restraining the actions of inflammatory mediators. A significant improvement in LPS-induced learning, memory and movement deficits in animal

models and considerable suppression of LPS induced oxidative stress, nitrosative stress, acetylcholinesterase activity and enhanced TNF-α levels was noticed. Among the 16-arylidene steroids, 16-(4-pyridylidene) steroid **1c** and its 4-aza analogue **6c** displayed highest potency and produced neuroprotective effects better than standard drugs celecoxib and dexamethasone. The studies indicate that the 16-aryldiene steroids should be extensively explored as promising drug candidates for the treatment of Alzheimer's and Parkinson's diseases consequent of neuroinflammatory mechanisms. The findings of this study may help to develop a novel and effective therapeutic intervention against neuroinflammation and neurodegenerative conditions.

METHODS

Chemistry

General. Melting points were determined on a Veego melting point apparatus and are uncorrected. IR (wave numbers in cm^{-1}) spectra are recorded on Perkin–Elmer spectrum RX 1, FT-IR spectrophotometer models using KBr pellets. ^{1}H NMR, ^{13}C NMR spectra were recorded on Bruker AC- 300F, 300 MHz using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) containing tetramethylsilane as internal standard (chemical shifts in ppm). Mass spectra were recorded on a Vg-11-250J 70 S model (VG Analytical Ltd., Manchester, England). Elemental analyses were carried out on a Perkin Elmer-2400 model CHN analyzer. Plates for TLC were prepared according to Stahl (E. Merck) using EtOAc as solvent (activated at 110 C for 30 min) and were visualized by exposure to iodine vapors. Anhydrous sodium sulphate was used as drying agent. All solvents were distilled prior to use according to standard procedure. The compounds were purified using flash chromatograph (Biotage, Isolera™ system, Sweden)

17β-Hydroxy-5-oxo-3,5-seco-4-norandrostan-3-oic acid (**3**), 3-Oxo-4-aza-androstan-17β-ol (**4**) and 4-aza-androstan-3,17-dione (**5**) were prepared using a reported procedure.[24]

*General procedure for the preparation of 16-arylidene steroids **1a-d***

A mixture of dehydroepiandrosterone (5.20 mmol), the appropriate aldehyde and sodium hydroxide (2.25 g, 56.25 mmol) in methanol (40 ml) was stirred at room temperature for 2 h until the reaction was completed (monitored by TLC). Cold water was added to the reaction mixture and the precipitate obtained was filtered, washed with water, dried and crystallized from methanol to obtain corresponding compounds **1a-d**.[22,23]

16-(2-Pyridylmethylene)-17-oxo-5-androsten-3β-ol (***1a***). Yield: 70%. White colour crystalline compound, mp 205-207 °C, mp lit.[22] 205-210 °C.

16-(3-Pyridylmethylene)-17-oxo-5-androsten-3β-ol (***1b***). Yield: 85%. White colour crystalline compound. mp 260-262 °C, mp lit.[22] 260-264 °C.

16-(4-Pyridylmethylene)-17-oxo-5-androsten-3β-ol (***1c***). Yield: 88%. Yellow colour crystalline compound. mp 219-221 °C, mp lit.[23] 220-224 °C.

16-(4-Methoxybenzylidene)-17-oxo-5-androsten-3β-ol (***1d***). Yield: 78%. Shining white colour crystalline compound. mp- 220-221 °C, mp lit.[22] 220 °C.

General procedure for the preparation of 4-aza-16-arylidene steroids ***6a-d***

A mixture of 4-azaandrostan-3,17-dione (0.5 g, 1.72 mmol), the requisite aldehyde and sodium hydroxide (2.25 g, 56.25 mmol) in methanol (40 ml) was stirred at room temperature for 2 h until the reaction was completed (monitored by TLC). Cold water was added to the reaction mixture and the precipitate obtained was filtered, washed with water, dried and crystallized from methanol to obtain corresponding 4-aza-16-benzylidene steroids **6a-h**.

16-(2-Pyridinylmethylene)-4-aza-androstan-3,17-dione (***6a***). Yield: 62%. Dark brown colour compound. mp 305-307 °C. FT-IR (KBr): 3374.01 (N-H), 2934.9 (C-H, aliphatic), 1715 (C=O), 1654.76 (CONH), 1365.86, 1092.45 (C-N) cm^{-1}. ^1H NMR: (400 MHz, DMSO-d_6) δ 0.62 (s, 3H, 18-CH_3), 0.65 (s, 3H, 19-CH_3), 2.98 (brs, 1H, 5α-*H*), 7.06 (s, 1H, vinylic-*H*), 7.11-7.14 (m, 1H, 5-C*H*, pyridine), 7.48 (d, J_o = 7.36 Hz, 1H, 6-C*H* , pyridine), 7.64 (t, J_o = 6.8 Hz, 1H, 4-C*H* , pyridine), 8.11 (s, 1H, N-*H*), 8.48 (s, 1H, 3-C*H*, pyridine). ^{13}C NMR:

(400 MHz, DMSO-d_6) δ 11.06 (**C**H_3), 14.5 (**C**H_3), 20.0 (**C**H_2), 26.6 (**C**H_2), 28.1 (**C**H_2), 28.4 (**C**H_2), 29.1 (**C**H_2), 31.0 (**C**H_2), 32.7 (**C**H_2), 33.6 (**C**H), 35.1 (**C**), 47.1 (**C**), 47.9 (**C**H), 50.5 (**C**H), 59.6 (**C**H), 123.3 (C=**C**-H), 126.7 (Ar**C**H), 130.4 (Ar**C**H), 136.8 (Ar**C**H), 139.7 (**C**=C-H), 149.9 (Ar**C**H), 154.1 (Ar**C**), 170.2 (**C**=O), 208.8 (**C**=O). MS: m/z 379.31 $[M+H]^+$. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 76.38; H, 7.73; N, 7.59.

*16-(3-Pyridinylmethylene)-4-aza-androstan-3,17-dione (**6b**)*. Yield 73%. Light brown colour compound. mp 298-300 °C. FT-IR (KBr): 3379.25 (N-H), 2936.12 (C-H, aliphatic), 1715.44 (C=O), 1659.52 (CONH), 1363.15, 1092.03 (C-N) cm^{-1}. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 0.90 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 3.20 (s, 1H, 5α-*H*), 7.22 (s, 1H, vinylic-*H*), 7.30 (brs, 1H, 6-C*H*, pyridine), 7.43-7.46 (m, 1H, 5-C*H*, pyridine), 8.00 (d, J = 8.04 Hz, 1H, N-*H*), 8.55 (d, J_m = 3.72 Hz, 1H, 4-C*H*, pyridine), 8.78 (d, J_m = 1.04, 1H, 2-C*H*, pyridine). ^{13}C NMR (400 MHz, DMSO-d_6): δ 11.07 (**C**H_3), 14.1 (**C**H_3), 20.0 (**C**H_2), 26.0 (**C**H_2), 28.0 (**C**H_2), 28.4 (**C**H_2), 28.6 (**C**H_2), 31.0 (**C**H_2), 32.7 (**C**H_2), 33.6 (**C**H), 35.1 (**C**), 47.1 (**C**), 48.1 (**C**H), 50.5 (**C**H), 59.6 (**C**H), 123.8 (C=**C**-H), 128.4 (Ar**C**H), 130.9 (Ar**C**), 136.6 (Ar**C**H), 138.3 (**C**=C-H), 149. (Ar**C**H), 151.2 (Ar**C**H), 170.2 (**C**=O), 207.9 (**C**=O). MS: m/z 379.31 $[M+H]^+$. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 75.90; H, 8.17; N, 7.28.

*16-(4-Pyridinylmethylene)-4-aza-androstan-3,17-dione (**6c**)*. Yield: 65%. White colour compound. mp 283-285 °C. FT-IR (KBr): 3363.12 (N-H), 2935.88 (C-H, aliphatic), 1716.84 (C=O), 1652.36 (COONH), 1363.5, 1016.21 (C-N) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.97 (s, 3H, 18-CH_3), 1.05 (s, 3H, 19-CH_3), 3.27 (s, 1H, 5α-*H*), 7.49 (s, 1H, vinylic-*H*), 8.12-8.16 (m, 1H, N-*H*), 8.29 (d, J_o= 6.28 Hz, 2H, 2-C*H* and 6-C*H*, pyridine), 9.0 (d, J_o=6.28 Hz, 2H, 3-C*H* and 5-C*H*, pyridine). ^{13}C NMR (400 MHz, DMSO-d_6) δ 11.06 (**C**H_3), 14.09 (**C**H_3), 19.9 (**C**H_2), 26.0 (**C**H_2), 28.03 (**C**H_2), 28.4 (**C**H_2), 28.6 (**C**H_2), 30.9 (**C**H_2), 32.7 (**C**H_2), 33.6 (**C**H), 35.1 (**C**), 47.2 (**C**), 47.9 (**C**H), 50.5 (**C**H), 59.6 (**C**H), 124.1 (C=**C**-H), 129.09 (2xAr**C**H), 140.8 (**C**=C-H), 142.1 (Ar**C**), 150.09 (2xAr**C**H), 170.1 (**C**=O) and 207.9

(**C**=O). MS: m/z 379.31 $[M+H]^+$. Anal. Calcd for $C_{24}H_{30}N_2O_2$: C, 76.16; H, 7.99; N, 7.40. Found: C, 76.29; H, 8.22; N, 7.33.

16-(4-Methoxybenzylidene)-4-aza-androstan-3,17-dione ***(6d)***. Yield: 55%. Yellow colour compound. mp 268-270 °C. FT-IR (KBr): 3329.68 (N-H), 2935.98 (C-H, aliphatic), 1706.94 (C=O), 1657.47 (CONH), 1254.27, 1174.69, 1025.65 (C-N) cm^{-1}. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 0.84 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 2.97 (d, J = 10.08 Hz, 1H, 5α-*H*), 3.80 (s, 3H, OCH_3), 7.02 (d, J_o= 8.40 Hz, 2H, 2-C*H* and 6-C*H*, aromatic), 7.25 (brs, 1H, vinylic-*H*), 7.59 (d, J_o = 8.48 Hz, 2H, 3-C*H* and 5-C*H*, aromatic), 7.70-7.76 (m, 1H, N-*H*). ^{13}C NMR: (400 MHz, DMSO-d_6) δ 11.06 (**C**H_3), 14.2 (**C**H_3), 20.06 (**C**H_2), 26.0 (**C**H_2), 28.1 (**C**H_2), 28.4 (**C**H_2), 28.6 (**C**H_2), 31.1 (**C**H_2), 32.5 (**C**H_2), 33.6 (**C**H), 35.1 (**C**), 46.9 (**C**), 48.2 (**C**H), 50.6 (**C**H), 55.2 (**C**-OCH_3), 59.6 (**C**H), 114.3 (2xAr**C**H), 127.6 (C=**C**-H), 131.7 (Ar**C**), 132.10 (2xAr**C**H), 133.6 (**C**=C-H), 160.1 (Ar**C**), 170.2 (**C**=O) and 208.3 (**C**=O). Anal. Calcd for $C_{26}H_{33}NO_3$: C, 76.62; H, 8.16; N, 3.44. Found: C, 76.35; H, 7.92; N, 3.22.

16-(4-Cyanobenzylidene)-4-aza-androstan-3,17-dione ***(6e)***. Yield: 75%. Brown colour compound. mp 300-304 °C. FT-IR (KBr): 3393.33 (N-H), 2937.28 (C-H, aliphatic), 2227.32 (C≡N), 1718.39 (C=O), 1637.83 (CONH), 1089.64 (C-N) cm^{-1}. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 0.88 (s, 3H, 19-CH_3), 0.92 (s, 3H, 18-CH_3), 3.02 (s, 1H, 5α-*H*), 7.28 (s, 1H, vinylic-*H*), 7.74-7.84 (m, 4H, 2-C*H*, 3-C*H*, 5-C*H* and 6-C*H*, aromatic), 7.94-7.96 (m, 1H, N-*H*). ^{13}C NMR: (400 MHz, DMSO-d_6) δ 11.0 (**C**H_3), 14.0 (**C**H_3), 19.9 (**C**H_2), 20.38 (**C**H_2), 25.9 (**C**H_2), 28.3 (**C**H_2), 28.7 (**C**H_2), 31.03 (**C**H_2), 32.8 (**C**H_2), 33.6 (**C**H), 35.04 (**C**), 47.1 (**C**), 48.1 (**C**H), 50.6 (**C**H), 59.7 (**C**H), 111.3 (Ar**C**), 118.3 (**C**≡N), 129.9 (C=**C**-H), 130.5 (2xAr**C**H), 132.2 (2xAr**C**H), 139.2 (Ar**C**), 139.6 (**C**=C-H), 170.3 (**C**=O) and 208.3 (**C**=O). MS: m/z 403.31 $[M + H]^+$. Anal. Calcd for $C_{26}H_{30}N_2O_2$: C, 77.58; H, 7.51; N, 6.96. Found: C, 77.79; H, 7.78; N, 7.20.

*16-(4-Nitrobenzylidene)-4-aza-androstan-3,17-dione (**6f**)*. Yield 55%. Dark brown colour compound. mp 238-240°C. FT-IR (KBr): 3386.61 (N-H), 2935.17 (C-H, aliphatic), 1720.14 (C=O), 1647.79 (CONH), 1518.53 and 1343.67 (NO_2), 1099.96 (C-N). ^{1}H NMR: (400 MHz, DMSO-d_6) δ 0.90 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 3.20 (s, 1H, 5α-*H*), 7.27-7.37 (m, 2H, vinylic-*H* and N-*H*), 7.87 (brs, 2H, 2-C*H* and 6-C*H*, aromatic), 8.25 (brs, 2H, 3-C*H* and 5-C*H*, aromatic). ^{13}C NMR: (400 MHz, DMSO-d_6) δ 13.9 (**C**H_3), 19.6 (**C**H_3), 20.4 (**C**H_2), 24.1 (**C**H_2), 25.5 (**C**H_2), 27.5 (**C**H_2), 28.8 (**C**H_2), 31.1 (**C**H_2), 31.7 (**C**H_2), 33.4 (**C**H), 33.6 (**C**), 47.0 (**C**), 48.0 (**C**H), 49.1 (**C**H), 57.9 (**C**H), 123.5 (2xAr**C**H), 129.3 (C=**C**-H), 131.00 (2xAr**C**H), 140.1 (Ar**C**), 141.6 (**C**=C-H), 146.9 (Ar**C**), 170.8 (**C**=O) and 207.8 (**C**=O). MS: m/z 423.33 $[M + H]^+$. Anal. Calcd for $C_{25}H_{30}N_2O_4$: C, 71.07; H, 7.16; N, 6.63. Found: C, 71.33; H, 6.90; N, 6.84.

*16-(4-Bromobenzylidene)-4-aza-androstan-3,17-dione (**6g**)*. Yield: 60%. Light yellow colour compound. mp- 285-287°C. FT-IR (KBr): 3270.83 (N-H), 2938.60 (C-H, aliphatic), 1720.5 (C=O), 1662.53 (CONH), 1395, 1073.20 (C-N). ^{1}HNMR (400 MHz, DMSO-d_6) δ 0.53 (s, 3H, 18-CH_3), 0.63 (s, 3H, 19-CH_3), 2,79 (s, 1H, 5α-*H*), 7.37-7.41 (m, 1H, vinylic-*H*), 7.42-7.47 (m, 2H, 2-C*H* and 6-C*H*, aromatic), 7.54 (m, 2H, 3-C*H* and 5-C*H*, aromatic), 8.03 (brs, 1H, N-*H*). ^{13}C NMR: (400 MHz, DMSO-d_6) δ 13.2 (**C**H_3), 14.1 (**C**H_3), 15.8 (**C**H_2), 20.3 (**C**H_2), 20.7 (**C**H_2), 25.4 (**C**H_2), 26.05 (**C**H_2), 28.3 (**C**H_2), 30.5 (**C**H_2), 30.8 (**C**H), 35.2 (**C**), 42.07 (**C**), 47.3 (**C**H), 47.4 (**C**H), 48.2 (**C**H), 123.9 (C=**C**-H), 126.2 (Ar**C**), 130.4 (2xAr**C**H), 131.9 (2xAr**C**H), 133..9 (Ar**C**), 141.6 (**C**=C-H), 170.5 (**C**=O) and 208.3 (**C**=O). Anal. Calcd for $C_{25}H_{30}BrNO_2$: C, 65.79; H, 6.63; N, 3.07. Found: C, 65.49; H, 6.84; N, 3.36.

*16-(2,3-Dimethylaminobenzylidene)-4-aza-androstan-3,17-dione (**6h**)*. Yield: 58%. Yellow colour compound. mp 255-257°C. FT-IR (KBr): 3376.38 (N-H), 3096.76 (C-H, aromatic), 2937.34 (C-H, aliphatic), 1723.8 (C=O), 1662.76 (CONH), 1521, 1346, 1050.36 (C-N). ^{1}HNMR: (400 MHz, $CDCl_3$) δ 0.91 (s, 3H, 18-CH_3), 1.13 (s, 3H, 19-CH_3), 3.09 (s, 7H, 5α-*H*

and N-(CH_3)$_2$), 6.70 (d, J_o = 7.8 Hz, 2H, 3-*CH* and 5-*CH*, aromatic), 6.97-7.11 (m, 1H, vinylic-*H*), 7.74 (d, J_o = 8.04 Hz, 2H, 2-*CH* and 6-*CH*, aromatic), 8.12 (brs, 1H, *N-H*). ^{13}C NMR: (400 MHz, $CDCl_3$) δ 13.6 (**C**H_3), 15.8 (**C**H_3), 18.2 (**C**H_2), 20.5 (**C**H_2), 21.6 (**C**H_2), 23.8 (**C**H_2), 28.6 (**C**H_2), 30.5 (**C**H_2), 31.1 (**C**H_2), 35.8 (**C**H_2), 36.1 (**C**), 40.1 (N(**C**H_3)$_2$), 47.4 (**C**), 51.01 (**C**), 55.4 (**C**H), 58.3 (**C**H), 111.0 (2xAr**C**H), 124.4 (C=**C**-H), 132.01 (2xAr**C**H), 138.1 (Ar**C**), 143.5 (**C**=C-H), 152.7 (Ar**C**), 190.3 (**C**=O) and 207.5 (**C**=O). Anal. Calcd for $C_{27}H_{36}N_2O_2$: C, 77.10; H, 8.63; N, 6.66. Found: C, 77.39; H, 8.89; N, 6.33.

Molecular Docking Studies

Docking was done using Glide (Schrodinger, LLC, New York, US). TNF-α crystal structure was downloaded from Protein Data Bank (PDB ID: 2AZ5). [37] The ligands were geometrically refined (cleaned) and conformers were generated with maximum number of conformers per structure as 1000 with force field OPLS-2005 with RMSD 1.0 A°. Protein was preprocessed, optimized and minimized with force field of OPLS2005 and RMSD of 0.30 Å using the protein preparation wizard. Grid was generated using the centroid of workspace of co-crystallized ligand that is composed of trifluoromethylphenyl indole and dimethylchromone moieties linked by a dimethylamine spacer that inhibited TNF-α receptor binding. The docking was performed using Glide with enabling the "write XP descriptor information" option and keeping the rest default.

Biological Activity

Animals. Male laca mice weighing 25-30 g and male wistar rats weighing 180-250 g (12-18 month old) were obtained from the central animal house of the institute. The animals were housed in standard laboratory conditions in groups of three at 25 ± 2°C, humidity of 60 ± 2% and 12 h light: Dark cycle. Animals had a free access to standard laboratory chow diet and tap water. The animals were acclimatized to the laboratory conditions 1 week prior to experimentation. All experiments were conducted daily between 09:00 am and 03.00 pm h.

The protocol was approved from Institutional Animal Ethics Committee and the animal housing, care and handling was followed as per committee guidelines for the purpose of control and supervision of experiments on animals and for animal care and use.

*Drug*s Lipopolysaccharide (LPS) (serotype E. *coli* 0111:B4) was procured from Sigma (St. Louis, MO, USA). Celecoxib and Dexamethasone were received as gift samples from M/s Panacea Biotech Ltd., Lalru. All other chemicals were of analytical grade.

Mice treated with LPS intraperitoneally: The mice were randomly divided into 18 groups, each group consisted of a minimum of 5 animals and daily received LPS as well as combination of LPS with standards (celecoxib and dexamethasone)[25,29,30] and synthesized compounds intraperitoneally (i.p.) for 5 days continuously 4h prior to training and testing sessions. The groups were divided as i) control group received equivalent volume of vehicles [distil water with 2 drops of tween 80 and 1% Phosphate Buffer Solution (PBS)] ii) LPS group (250µg/kg) iii) combination of LPS (250µg/kg) with celecoxib (20 mg/kg) iv) combination of LPS (250µg/kg) with dexamethasone (5 mg/kg) and v-xviii) combination of LPS (250µg/kg) with synthesized compounds **1a-d** and **6a-h** (5 mg/kg). The behavioural assessment after intraperitoneal injection of LPS in mice has been carried out using Morris water maze and Elevated plus maze.

Morris water maze- The mice were evaluated using a spatial version of the Morris water maze model[29] for 5 days. The Morris water maze for mice consisted of a circular pool (60 cm in diameter, 25 cm in height) filled to a depth of 20 cm with water maintained at 25°C. The tank was located in a large room and water was made opaque with nontoxic white colored dye. The tank was divided into four equal quadrants with the help of two threads, fixed at right angle to each other on the rim of the pool. A submerged platform (with top surface 6 x 6 cm) was placed inside the target quadrants (Q4 in present study) of this pool 1 cm below surface of water and position of platform was kept unaltered throughout the

training session. The mice received four consecutive daily training trials (day 1 to day 4), with each trial having a total limit of 110 s and a next trial interval of approximately 30 s. During the training session, the mice were placed into the water with their heads facing the wall at one of four starting positions (sequence of which was selected randomly) and allowed 90 s to locate submerged platform. If the mice failed to find the platform within 90 s, it was guided gently on to the platform and allowed to remain there for 20 s. On the 5th day, escape latency (the time taken by the animal to move from the starting quadrant to find the hidden platform in the target quadrant) was measured along with mean time spent in the target quadrant (TSTQ) that was index of retrieval or memory.

Elevated plus maze- Memory acquisition and retention was evaluated using the elevated plus maze test at day 5 of experimental protocol.[29] The elevated plus maze for mice consisted of two open arms (16 x5 cm) and two covered arms (16 x 5 x15 cm) extended from a central platform (5 x 5 cm) and the maze was elevated to a height of 25 cm from the floor. In training session, each mice was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) is the time taken by the animal to move from the open arm into one of the covered arms with all its four legs. TL was recorded on the training session (i.e. 4th day of drug administration) for each animal. If the animal did not enter into one of the covered arm within 90 s, it was gently pushed into one of the two covered arms and TL was assigned as 90 s. The mice were allowed to explore the maze for another 30 s and then returned to their home cage. Retention of this learned task (memory) was examined after 24 h (5th day) of the training session. The results are expressed in terms of percentage initial transfer latency (% ITL).

Rats treated with LPS intranigrally: The stereotaxic surgery was performed on the rats anaesthetized with thiopentone (45 mg/kg, i.p, Neon Laboratories, India). The head was positioned in a stereotaxic frame and a midline sagittal incision was made in the scalp. Burr

holes were drilled in the skull on left sides using the following coordinates: 5.5 mm posterior, 1.5 mm lateral and 8.3 mm ventral to the bregma. The coordinates for the placement of cannulae were determined by using atlas of Paxinos and Watson (2007).[25] LPS [10 µg in 2µl of 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M); pH 7.4)] was infused as a single intranigral injection into the left substantia nigra using the Hamilton micro syringe. The skin was sutured after the injection followed by daily application of antiseptic powder (Neosporin®).The rats were randomly divided into 19 groups, each group consisted of a minimum of 5 animals. The animals of all the groups received single intranigral injection of LPS (10 µg in 2µl of PBS) on day 1 and daily intraperitoneal (i.p.) injection of standards (celecoxib (20 mg/kg, i.p), dexamethasone (2 mg/kg, i.p) or synthesized compounds (2 mg/kg, i.p) continuously for 21 days. The groups were divided as i) sham group received intranigral injection of PBS and equivalent volume of normal saline (0.9% NaCl, i.p.) ii) LPS + vehicle group received intranigral injection of LPS and equivalent volume of vehicle (i.p.) iii) LPS group received intranigral injection of LPS iv) celecoxib group received intranigral injection of LPS and celecoxib (20 mg/kg, i.p.) v) dexamethasone group received intranigral injection of LPS and dexamethasone (2 mg/kg, i.p.) vi-xix) intranigral injection of LPS and synthesized compounds **1a-d** and **6a-h** (2 mg/kg, i.p). The behavioural assessment after intranigral injection of LPS has been carried out using actophotometer, elevated plus maze and block tests on 7^{th}, 14^{th} and 21^{st} day. As there was no significant difference in the behavioural observation of animals at day 7 and 14, the behavioural data of day 21 is presented.

The actophotometer (IMCORP, India) was used to evaluate the locomotor activity. Rats were individually placed in photometer and the total activity count was registered for 5 min. Each animal was observed in a square (30 cm) of closed arena equipped with infrared light sensitive photocells, using a digital photoactometer, values are expressed as counts per

5 min. The apparatus was placed in a dark, light and sound attenuated and ventilated testing room.[38]

The memory acquisition and retention was tested using the elevated plus maze test. The apparatus consisted of two crossed arms, one closed and the other open. Each animal was placed on the open arm facing outwards.[29] The time taken by the animal to enter the closed arm in the first trial (acquisition trial) on 6th day was noted and was designated the initial transfer latency. Cut-off time was fixed as 90 s and in case an animal could not find the closed arm within this period, it was gently pushed in to one of the closed arms and allowed to explore the maze for 30 s. The final trial (retention trial) was performed on 21st day and retention transfer latency was noted. The retention trial latency is expressed as percentage of initial trial latency.

Catatonia was measured using wooden block test method. The development and severity of the four stages of catatonia were observed and scored as follows: Stage 1, rat moves when placed on the table, score = 0; Stage 2, rat moves only when touched or pushed, score = 0.5; Stage 3, rat placed on the table with front paws set alternately on a 3 cm high block fails to correct the posture in 10 s, score = 0.5 for each paw with a total of 1 for this stage; State 4, rat fails to move when the front paws are placed alternately on a 9 cm high block, score = 1 for each paw with a total score 2 for this stage. Thus, the maximum possible score would be 3.5 reflecting full catatonia. Lesser score would mean an apparently lesser degree of catatonia.[39]

Estimations of biochemical parameters in brain supernatants of mice and rats: The brain homogenates were prepared on day 5 for mice and day 21 for rats. The mice (n=5) and rats (n=5) were sacrificed under deep anaesthesia and brains were rapidly removed and placed on dry ice for isolation of the cerebral cortex and hippocampus. A 10% (w/v) tissue (cortex and hippocampus) homogenate was prepared in chilled 0.1M phosphate buffer (pH 7.4) using a

Potter Elvenhjem homogenizer. The homogenate was centrifuged at 12000 rpm for 20 min at 4^0C to obtain the post mitochondrial supernatant (PMS), which was used for further biochemical analysis. The biochemical parameters evaluated include:-

i) Protein Estimation: The protein content was measured using bovine serum albumin as standard.[40]

ii) Estimation of Acetylcholinesterase Activity (AChE Assay): Cholinergic dysfunction was assessed by measuring acetylcholinesterase (AChE) levels in PMS according to the method of Ellman et al. (1961).[41] Results were calculated using molar extinction coefficient of chromophore (1.36 x 104 $M^{-1}cm^{-1}$) and expressed as percentage of sham group.

iii) Estimation of Lipid Peroxidation (LPO Assay): The malondialdehyde content, a measure of lipid peroxidation (LPO) was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills (1965).[42] The 0.5 ml of PMS and 0.5 ml of Tris–HCl were incubated at 37 ^{0}C for 2 h. After incubation 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000 g for 10 min. Then 1 ml of 0.67% thiobarbituric acid was added to 1 ml of supernatant and the tubes were kept in boiling water for 10 min. After cooling, 1 ml double-distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56 x 105 M^{-1} cm^1 and expressed as n moles of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method and the brain malondialdehyde content expressed as n moles of MDA/mg protein.

iv) Plasma Nitrite Estimation (Nitrite Assay): Nitrite was estimated in the PMS using the Greiss reagent and served as an indicator of nitric oxide production. A measure of 500 ml of Greiss reagent (1:1 solution of 1 % sulphanilamide in 5 % phosphoric acid and 0.1% napthaylaminediamine dihydrochloric acid in water) was added to 100 ml of post-mitochondrial supernatant and absorbance was measured at 546 nm.[43] Nitrite concentration

was calculated using a standard curve for sodium nitrite and nitrite levels are expressed as mg/ml. Although, the Griess spectrophotometric assay is not a leading methodology for the quantification of nitric oxide, it employs an indirect measure of nitric oxide content.

v) Estimation of reduced glutathione (GSH Assay): Reduced glutathione (GSH) was assayed by the method of Jollow et al. (1974).[44] The 1.0 ml of PMS was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4^0C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4^0C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm and the reduced glutathione levels were expressed as nmol of GSH/mg protein.

vi) Estimation of superoxide dismutase (SOD Assay): Superoxide dismutase (SOD) activity was assayed by the method of Kono (1978).[45] The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of Nitro-blue tetrazolium (NBT). In a cuvette, 2 ml of the above mixture was taken and 0.05 ml of PMS and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added to it. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30-/60-s intervals.

vii) Estimation of Catalase (Catalase Assay): Catalase activity was assayed by the method of Claiborne (1985).[46] Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml PMS in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated and expressed μmoles of H_2O_2 decomposed/min/mg protein.

Inhibition of TNF-α: Quantification of TNF-α (R&D Systems Quantikine Rat TNF-α immunoassay kit) was made according to the manufacturer's instructions. The Quantikine

Rat TNF-α immunoassay is a 4.5-h solid phase ELISA designed to measure rat TNF-α levels. For the assay the blood samples were collected from retro orbital sinus of rat on 21st day and subjected to centrifugation at 10000 rpm for 10 min. The serum thus obtained was used for the assay. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α was pre-coated in the microplate. The rat serum sample of different groups (n=5) were pipetted into the wells and rat TNF-α was bound by the immobilized antibody. After washing any unbound substance, an enzyme-linked polyclonal antibody specific for rat TNF-α was added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the colour was measured at 450 nm in proportion to the amount of rat TNF-α bound. The sample values were then read off using standard curve. Values were expressed as mean ± SEM.[34]

Data analysis All statistical analyses were performed using GraphPad Prism v. 5.03 software (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± SEM. The intergroup variation in behavioural, biochemical and molecular estimations were measured by one way and two way analysis of variance (ANOVA) followed by Turkey's test. A value of $p < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

The financial support provided by the University Grant Commission (UGC), New Delhi, India for financial assistance under Research Fellowship in Science for Meritorious Student (RFSMS) Scheme is gratefully acknowledged.

Supporting Information

Copies of ^{1}H NMR and ^{13}C NMR spectra of compounds **6a-h** and Mass spectra of compounds **6a-c, 6e, 6f** and **6h**. This material is available free of charge via the Internet at http://pubs.acs.org.

ABBREVIATIONS

AD, Alzheimer's disease; PD, Parkinson's disease; LPS, lipopolysaccharide; DHEA, dehydroepiandrosterone; CNS, central nervous system; Aβ, amyloid-β; APP, amyloid precursor protein; IL, interleukin; TNF-α, tumor necrosis factor; NMDA, N-methyl-D-aspartate; TSTQ, time spent in target quadrant; % ITL, initial transfer latency; ANOVA, analysis of variance; S.E.M, standard error mean; AchE, acetylcholinesterase; LPO, lipid peroxidation; GSH, Glutathione; SOD, superoxide dismutase; SN, substantia nigra; COX, cyclooxygenase; PBS, phosphate buffer solution.

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Table of Contents graphic