

Lupeol Isolated from *Betula alnoides* Ameliorates Amyloid Beta Induced Neuronal Damage via Targeting Various Pathological Events and Alteration in Neurotransmitter Levels in Rat's Brain

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Abstract

Lupeol, a natural active constituent of *Betula alnoides* (BA), is well known for its anti-inflammatory, anti-oxidant and neuroprotective activities. In present study the therapeutic potential of lupeol was investigated against amyloid beta ($A\beta_{(1-42)}$) induced cognitive deficit, neurochemical and biochemical abnormalities in rats. Lupeol was isolated from the BA and its structure was confirmed through nuclear magnetic resonance spectra. $A\beta_{(1-42)}$ (4 $\mu\text{g}/4 \mu\text{L}$) intracerebroventrically (icv) was administered to rats for the induction of Alzheimer's disease (AD). Lupeol treatment (25 mg/kg/day, 50 mg/kg/day and 100 mg/kg/day per orally) was started one week after $A\beta_{(1-42)}$ infusion up to the 21st days. Morris water maze from day 16 to 21 and object recognition tasks on day 14 and 15 were performed for memory assessment. On 22nd day, animals were sacrificed and hippocampi were isolated for analysis of biochemical (acetylcholinesterase, lipid hydroperoxide, glutathione and nitrite) and neuro-inflammatory (tumor necrosis factor $-\alpha$, interleukin (IL)-1 β , and IL-6) parameters. In the present study $A\beta_{(1-42)}$ infusion was significantly impaired behavioral memory, increased oxidative stress, decreased antioxidant enzyme and increased pro-inflammatory markers. Treatment of lupeol significantly restored $A\beta_{(1-42)}$ induced behavioral and biochemical abnormalities in rats brain. The findings of the present study suggest that lupeol act through multiple mechanisms and would be used to curb cognitive decline associated with neurodegenerative disorders of AD.

Keywords: Alzheimer's disease; Lupeol; Memory; Cognitive disorder; Oxidative stress

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Introduction

Alzheimer's disease (AD) is the most common form of dementia characterized by extracellular deposits of amyloid beta ($A\beta_{(1-42)}$), intracellular neuro-fibrillary tau tangle, oxidative stress and decline in cognitive functions [1-3]. Although the exact pathogenic mechanisms remain unclear, transcriptional dysregulation and impaired cyclic nucleotide signaling have been reported in experimental animals as well as in AD patients [4-6]. Recent neuropathological studies have also established a link between morphological and functional changes occurring in the monoaminergic ascending system, particularly in nor

epinephrine (NE) and 5-hydroxytryptamine (5-HT), and the pathophysiology of AD. Moreover clinical researchers found that AD patients have complex neurochemical disturbances including the catecholaminergic, cholinergic and glutaminergic neuronal systems [7-10].

Lupeol, a biologically active dietary triterpenoid is found in many medicinal plants and different fruits such as olives, mangoes, and strawberries [11,12]. A variety of medicinal plants such as *Betula alnoides*, *Vernonanthura ferruginea* and *Zanthoxylum rhoifolium* have also been reported to contain lupeol as an active constituent [13-15]. Lupeol has been reported to have various pharmacological activities including acetylcholinesterase (AChE)

inhibition, anti-inflammatory and anti-oxidant actions [16-18]. Further, it has been reported to be effective in various pathologies and recently its neuroprotective effect has been studied [19,20]. Indeed Brimson et al. [21] described a neuroprotective effect of lupeol against glutamate-induced neurotoxicity in HT22 mouse hippocampal cells.

Intra-cerebroventricular (ICV) administration of $A\beta_{(1-42)}$ is a well-known model to produce behavioral, biochemical and neuropathological changes similar to that seen in clinical AD and considered as an appropriate animal model of AD [22]. Thus, the present work was carried out to investigate the neuroprotective potential of lupeol against ICV- $A\beta_{(1-42)}$ induced cognitive impairment, neurotransmitters deficits, neuroinflammation and oxidative-nitrosative stress in rats.

Material and Methods

Drugs and chemicals

The stem bark of *Betula alnoides* (BA) was collected from the mountains of Himachal Pradesh, INDIA. The plant BA, was identified by Dr. H.B. Singh, an eminent botanist (Ref. No.-NISCAIR/ RHMD/ CONSULT/ 2014/ 2398-178) of NISCAIR, New Delhi, India. $A\beta_{(1-42)}$ was purchased from Sigma-Aldrich, USA. All other chemicals used in the study were of analytical grade. Solutions of the drugs and chemicals were always prepared afresh before use.

Extraction and fractionation of BA (Stem bark)

50 gm of dried and powdered bark of BA was soxhlated with ethanol (60°C to 80°C) yielding 14.6 g of dry ethanolic extract. Lupeol, as a major constituent was separated out using a column chromatography technique [23]. Different spectroscopic methods were used to elucidate the structure of lupeol using Fourier transform infrared spectroscopy (FTIR) -Nicolet S10 (Thermospecific) in CCl_4 at I.S.F. Analytical Lab, Moga. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE-400 Japan (100 MHz and 400 MHz) in chloroform with tetramethylsilane as internal standard at the SAIF, Punjab University, Chandigarh, India.

Animals

Male wistar rats (250-300 g) were obtained from Central Animal House of I.S.F. College of Pharmacy, Moga, Punjab (India). Animals were kept in polyacrylic cages (4/cage) and maintained under standard husbandry conditions (room temperature $22 \pm 1^\circ C$ and relative humidity of 60%) with a 12 h light/dark cycle (lights on at 8 AM). The protocol was reviewed and approved by the Institutional Animal Ethics Committee and the experiments were carried out according to Indian National Science Academy guidelines.

Experimental procedure

Animals were anesthetized with ketamine (100 mg/kg, i.p) and xylazine (5 mg/kg, i.p) and were fixed on stereotaxic apparatus. Skull was exposed after midline sagittal incision and holes on both sides of the brain were drilled according to Paxinos and

Watson [24]. Animals were divided into five groups and each group comprised of 10 animals. The treatment schedule and the interval for estimation of various parameters were described as; Group1: served as double vehicle control, received artificial cerebrospinal fluid (ACSF) in a volume of 4 μL in each ventricle on day 1 and 2% dimethyl sulfoxide per orally (p.o., as a vehicle of lupeol) for 21 days. Group 2: Rats were infused with ICV- $A\beta_{(1-42)}$ (4 $\mu g/4 \mu L$) dissolved in ACSF in a volume of 4 μL in each ventricle on day 1. Group 3, 4 and 5 received lupeol at doses of 25 mg/kg, 50 mg/kg, 100 mg/kg p.o. respectively 1 week after the ICV- $A\beta_{(1-42)}$ infusion starting from day 7 and continued once daily at 10 am daily for a period of 21 days. The doses of lupeol were selected on the basis of earlier reports in which significant antioxidant and neuroprotective properties were demonstrated [18,25].

Object Recognition test (ORT): The ORT was performed as described by Giorgetti et al. [26].

Spatial navigation task: Spatial learning and memory of animals in a Morris water maze (MWM) was tested by the method described by Morris [27].

Brain homogenate preparation: Terminally, on day 22, rats were sacrificed and hippocampus from the brain was separated, weighed and then homogenized individually. The various biochemical parameters were determined separately in hippocampal supernatant collected following homogenization. Animals were perfused with phosphate buffer saline before decapitation from the heart to remove blood from the brain tissues completely and its interference with the homogenate readouts. After perfusion, animals were sacrificed by decapitation and brains were removed and rinsed with ice-cold isotonic saline. The rat hippocampal tissues were then homogenized with ice cold 0.1 M phosphate buffer (pH 7.4) in a volume 10 times the weight of the tissue. The homogenate was centrifuged at 10,000 g for 15 min ($4^\circ C$) and aliquots of supernatant were separated and used for biochemical analysis. Protein was measured in all brain samples by the method of Lowry et al. [28] using bovine serum albumin (1 mg/ml) as a standard.

AChE activity: The quantitative measurement of AChE activity in brain was performed according to the method described by Ellman et al. [29].

Malondialdehyde (MDA) estimation: The quantitative measurement of MDA end product of lipid peroxidation-in brain homogenate was performed according to the method of Wills [30].

Nitrite estimation: The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay using Greiss reagent (0.1% N-[1-naphthyl] ethylene diaminedihydro chloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green et al. [31].

Reduced glutathione (GSH) estimation: GSH in hippocampus was estimated according to the method described by Ellman [32].

Pro-inflammatory cytokines such as tumor necrosis factor $-\alpha$ (TNF- α), interleukin (IL)-1 β and IL-6 estimation: The quantifications of TNF- α , IL-6, and IL-1 β were done by rat TNF- α , IL-6, and IL-1 β immunoassay kit (KRISHGEN BioSystem, Ashley Ct, Whitti-

er, CA). Concentrations of TNF- α , IL-6, and IL-1 β were calculated from the plotted standard curves [33].

Statistical analysis

The values were expressed as mean \pm standard error mean (SEM). $P < 0.05$ was set to be statistically significant. The results were analyzed using Analysis of variance using statistical Graph Pad Prism software. In ORT, time of exploration during T1 and T2 on familiar and novel object was analyzed by paired student t-test.

Results

Isolation of lupeol

The melting point of the compound, lupeol was found to be 213°C to 215°C. The FTIR spectra gave information about the functional groups or chemical entities present in lupeol which was found in accordance with the literature data of lupeol [33]. Further 1H NMR spectrum of the compound revealed the presence of seven tertiary methyl protons, a sextet of one proton at δ 2.17 and presence of olefinic protons at (H-29a and b) which is characteristic of lupeol [34]. On the basis of FTIR and NMR spectra structure of compound was drawn which was in good conformity for the structure of lupeol (Figure 1).

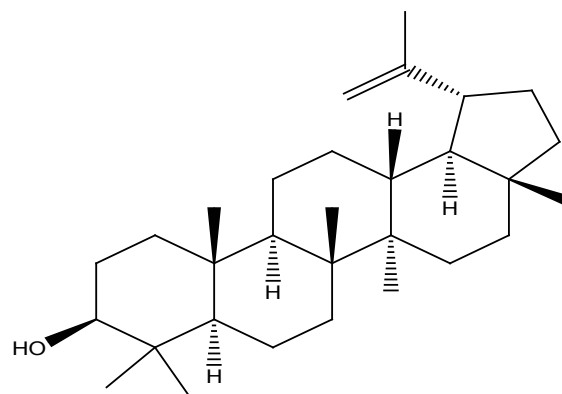


Figure 1 Chemical structure of lupeol.

Effect of lupeol on memory performance in MWM task of ICV-A β (1-42)-infused rats

In the MWM, animals were trained for 5 days starts on day 17 of A β (1-42)-infusion. On day 17 (1st trial), there was no significant difference between the mean latencies of all groups. A β (1-42)-infused animal showed poor ability (increased latency to find the platform) to learn the task on days 18, 19 and 20 as compared to control group. On 21st day the time spent in the target quadrant were also significantly decreased in A β (1-42)-infused animal as compared to control group (Figure 2A). A β (1-42)-infused rats failed to remember the precise location of the platform, spent significantly less time in the target quadrant as compared with control group (Figure 2B). Whereas pretreatment with lupeol (25, 50 and 100 mg/kg) significantly attenuated A β (1-42)-induced acquisition deficit and showed a significant difference between the mean latencies.

Effect of lupeol on non-spatial memory performance in ORT in ICV- A β (1-42)-infused rats

There was no significant difference observed between the A β (1-42)-infused and Lupeol-treated rats for familiar objects (Figure 3A). When A β (1-42) infused rats were exposed to familiar and novel objects in ORT, they were unable to discriminate between familiar and novel objects and spend almost equal time to explore the similar and novel objects as compared to control group (Figure 3B). Whereas, lupeol (25 mg/kg, 50 mg/kg and

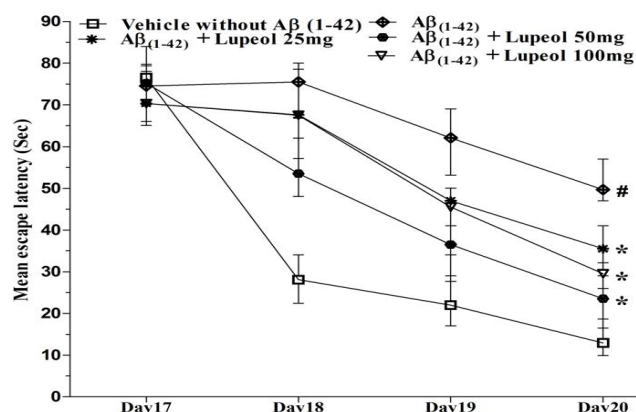


Figure 2A Effect of lupeol on MWM performance (mean escape latency) in A β (1-42) infused rats. The values are expressed as mean \pm SEM (n=10). (A & B) # $P < 0.001$ vs. vehicle, * $P < 0.05$ vs. A β (1-42)*

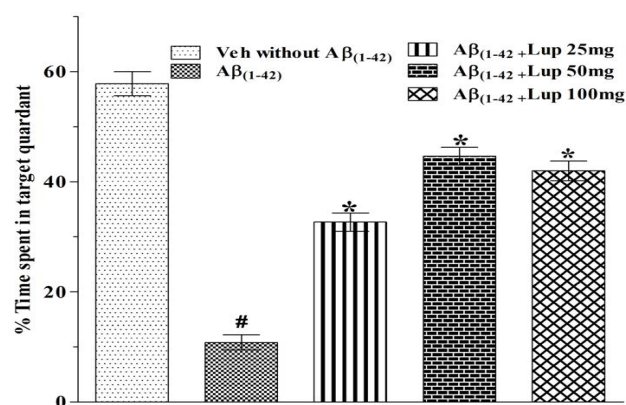


Figure 2B Effect of lupeol on MWM performance (time spend in target quadrant) in A β (1-42) infused rats. The values are expressed as mean \pm SEM (n=10). (A & B) # $P < 0.001$ vs. vehicle, * $P < 0.05$ vs. A β (1-42)*

100 mg/kg) treatment produced significant improvement in discriminating ability between familiar and novel object in $A\beta_{(1-42)}$ infused rats (Figure 3C).

Effect of lupeol on AChE activity in $A\beta_{(1-42)}$ -infused rats

ICV infusion of $A\beta_{(1-42)}$ produced significant increase in AChE activity as compared with vehicle treated groups. Lupeol (25 mg/kg, 50 mg/kg and 100 mg/kg) attenuated $A\beta_{(1-42)}$ -induced elevation in AChE activity (Figure 4) and restored the values as that of vehicle control rats.

Effect of lupeol treatment on brain oxidative-nitrosative stress (MDA, GSH and nitrite) levels in ICV- $A\beta_{(1-42)}$ injected rats

The level of MDA and nitrite were increased and GSH was decreased significantly in $A\beta_{(1-42)}$ infused groups as compared of control group. Lupeol (25 mg/kg, 50 mg/kg and 100 mg/kg) treatment significantly restored the MDA, nitrite and GSH levels as compared with those of $A\beta_{(1-42)}$ -infused rats (Table 1).

Effect of lupeol treatment on brain pro-inflammatory cytokines levels in ICV- $A\beta_{(1-42)}$ -infused rats

The levels of TNF- α , IL-6 and IL-1 β were increased significantly in hippocampus following $A\beta_{(1-42)}$ infusion as compared with control group. Lupeol (25 mg/kg, 50 mg/kg and 100 mg/kg) treatment significantly restored the hippocampal pro-inflammatory cytokines level as compared with $A\beta_{(1-42)}$ infused rats (Table 2).

Discussion

The present study demonstrates the neuroprotective potential of lupeol against $A\beta_{(1-42)}$ induced behavioral, biochemical and neurochemical abnormalities in rats. ICV infusion of $A\beta_{(1-42)}$ in rats is known to produce cognitive deficit and other neuropathological changes similar to those seen in AD and thus considered as

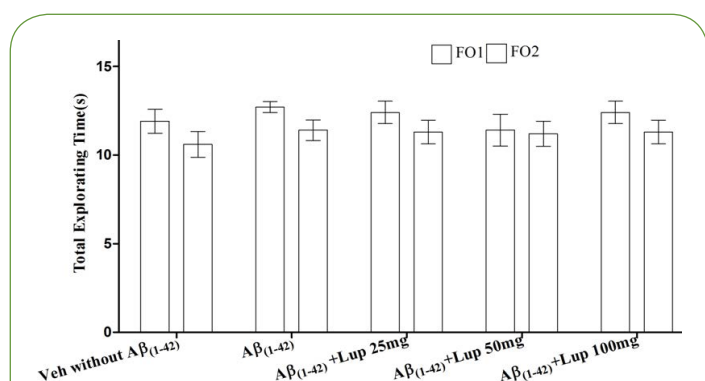


Figure 3A Effect of lupeol in ORT in $A\beta_{(1-42)}$ infused rats. The values are expressed as mean \pm SEM (n=10). (A) The performance of rats in (T1) sample phase trial with two similar objects (FO1 and FO2). No significant difference was observed between the untreated and Lupeol-treated rats.

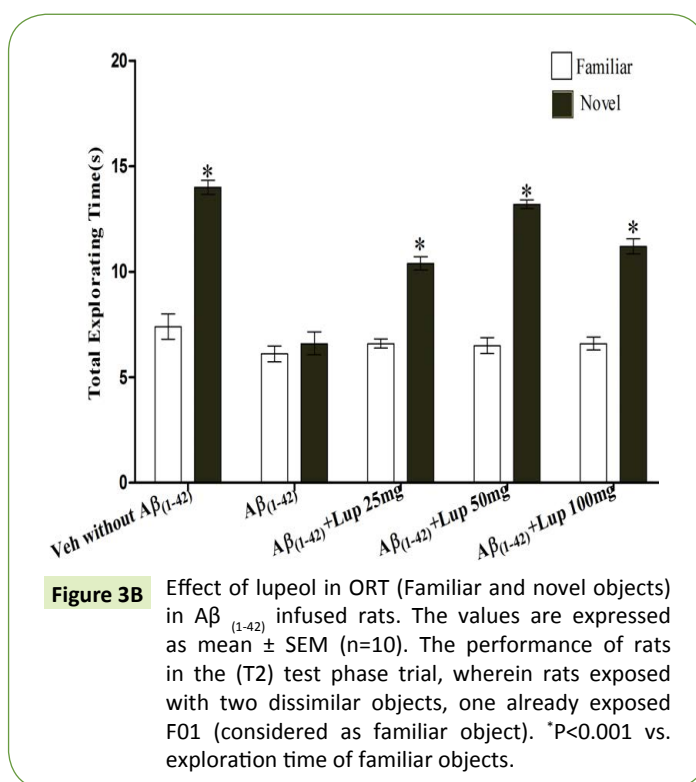


Figure 3B Effect of lupeol in ORT (Familiar and novel objects) in $A\beta_{(1-42)}$ infused rats. The values are expressed as mean \pm SEM (n=10). The performance of rats in the (T2) test phase trial, wherein rats exposed with two dissimilar objects, one already exposed FO1 (considered as familiar object). * $P < 0.001$ vs. exploration time of familiar objects.

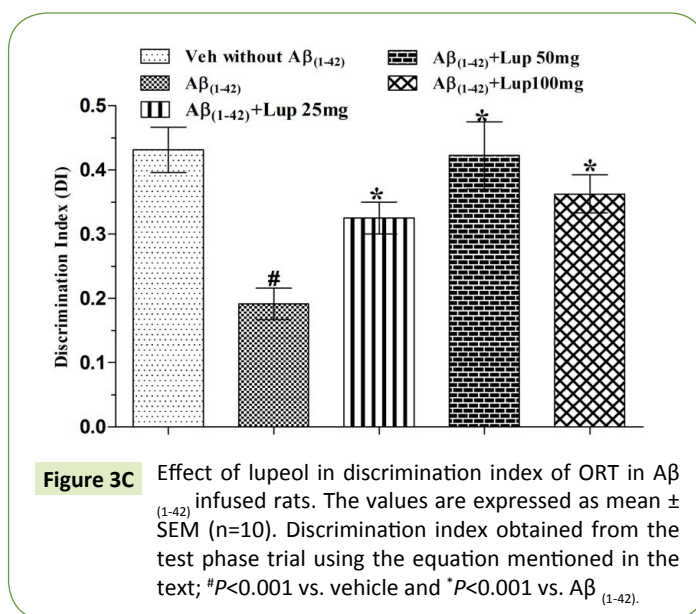


Figure 3C Effect of lupeol in discrimination index of ORT in $A\beta_{(1-42)}$ infused rats. The values are expressed as mean \pm SEM (n=10). Discrimination index obtained from the test phase trial using the equation mentioned in the text; # $P < 0.001$ vs. vehicle and * $P < 0.001$ vs. $A\beta_{(1-42)}$.

a suitable model system of AD [23,35]. In the present study, ICV- $A\beta_{(1-42)}$ infusion produced cognitive impairment, oxidative stress, neuroinflammation and hippocampal neurotransmitter deficit demonstrating similar changes following $A\beta_{(1-42)}$ infusion in rats [35]. $A\beta_{(1-42)}$ is an oligomer, reported to cause destruction in the selective brain regions such as hippocampus, cortex and perirhinal cortex that affects spatial and non-spatial memory respectively [36-38]. MWM and ORT were used to assess spatial and non-spatial memory respectively. In the present study, $A\beta_{(1-42)}$ infused rat showed poor learning and consolidation of memory in MWM task. In addition, these animals were unable to discriminate between novel and familiar objects indicating

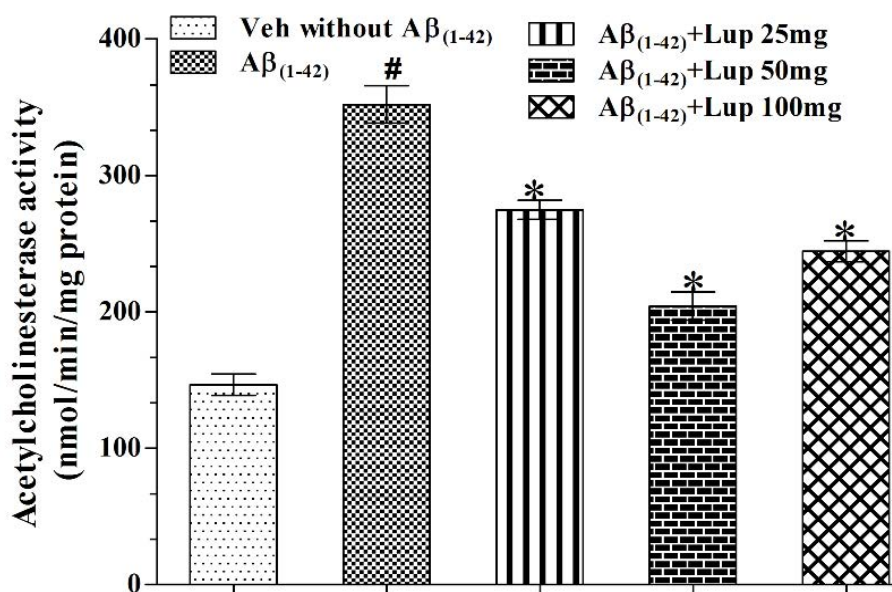


Figure 4 Effect of lupeol on AChE activity in Aβ₍₁₋₄₂₎ infused rats. The values are expressed as mean ± SEM (n=10). [#]P<0.001 vs. Vehicle; ^{*}P<0.001 vs. Aβ₍₁₋₄₂₎.

Table 1 Effect of lupeol on rat hippocampal oxidative-nitrosative stress parameters in Aβ₍₁₋₄₂₎-infused rats.

Groups	Biochemical Parameters		
	Nitrite (μmol/mg protein)	MDA (nmol/mg protein)	GSH (μmol /mg protein)
Vehicle without Aβ ₍₁₋₄₂₎	85.8 ± 08.63	1.075 ± 0.344	0.950 ± 0.051
Aβ ₍₁₋₄₂₎	208.6 ± 16.92 [#]	5.213 ± 0.143 [#]	0.175 ± 0.040 [#]
Aβ ₍₁₋₄₂₎ + Lupeol 25 mg	178.9 ± 15.87 [*]	3.813 ± 0.591 [*]	0.485 ± 0.034 [*]
Aβ ₍₁₋₄₂₎ + Lupeol 50 mg	139.0 ± 14.88 [*]	2.913 ± 0.779 [*]	0.625 ± 0.043 [*]
Aβ ₍₁₋₄₂₎ + Lupeol 100 mg	148.9 ± 19.37 [*]	3.313 ± 0.591 [*]	0.535 ± 0.034 [*]

The values were expressed as mean ± SEM. [#]P<0.001 vs. Vehicle; ^{*}P<0.001 vs. Aβ₍₁₋₄₂₎.

The values were expressed as mean ± SEM The results were analyzed using two-way ANOVA

followed by Bonferonni test for multiple comparisons and one way ANOVA followed by

Tukey's test, for multiple groups using statistical Graph Pad Prism software (version 5.0, La Jolla, CA, USA). [@]P<0.001 vs. Vehicle; ^{*}P<0.001 vs. Aβ₍₁₋₄₂₎.

Table 2 Effect of lupeol on rat hippocampal TNF-a, IL-6, and IL-1b levels in Aβ₍₁₋₄₂₎ rats.

Groups	Biochemical Parameters		
	TNFα (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
Vehicle without Aβ ₍₁₋₄₂₎	43.31 ± 6.717	26.10 ± 4.990	22.17 ± 0.799
Aβ ₍₁₋₄₂₎	107.20 ± 9.058 [#]	88.10 ± 8.751 [#]	71.15 ± 7.751 [#]
Aβ ₍₁₋₄₂₎ + Lupeol 25 mg	81.11 ± 6.072 [*]	62.16 ± 4.930 [*]	56.80 ± 7.930 [*]
Aβ ₍₁₋₄₂₎ + Lupeol 50 mg	68.79 ± 6.820 [*]	51.93 ± 6.085 [*]	39.46 ± 6.085 [*]
Aβ ₍₁₋₄₂₎ + Lupeol 100 mg	74.41 ± 8.392 [*]	57.96 ± 5.930 [*]	47.80 ± 7.930 [*]

The values were expressed as mean ± SEM. [#]P<0.001 vs. Vehicle; ^{*}P<0.001 vs. Aβ₍₁₋₄₂₎.

The values were expressed as mean ± SEM The results were analyzed using two-way ANOVA followed by Bonferonni test for multiple comparisons

and one way ANOVA followed by Tukey's test, for multiple groups using statistical Graph Pad Prism software (version 5.0, La Jolla, CA, USA). [@]

P<0.001 vs. Vehicle; ^{*}P<0.001 vs. Aβ₍₁₋₄₂₎.

cognitive impairment which was significantly restored by lupeol treatment [39,40].

Recent neuropathological studies have found that AD patients have complex neurochemical disturbances including the catecholaminergic, cholinergic neuronal systems and their restoration may improve memory in AD [9,10]. In addition, modulators of acetylcholine (ACh), dopamine, 5-HT and NE have also been reported to improve hippocampal dependant memory in clinical and preclinical cases of AD [41-43]. $A\beta_{(1-42)}$ in both clinical and preclinical AD, has reports to causes catecholaminergic deficit by escalating AChE enzymatic activity [44,45]. In agreement with the previous findings, the present study also showed significant increase in AChE activity in $A\beta_{(1-42)}$ infused brains [6-10]. However, the treatment of lupeol was able to significantly decrease AChE activity as reported earlier [17]. The finding of the current study suggest that lupeol contributes to the restoration of ACh level by reducing the level of AChE in $A\beta_{(1-42)}$ infused rat's hippocampus. Thus, recovery in the levels of these monoamines may contribute to the beneficial effects of lupeol on learning and memory in $A\beta$ induced AD in rats.

Neuroinflammation and oxidative-nitrosative stress are two most common events of $A\beta_{(1-42)}$ -induced neurotoxicity and neuronal cell death as seen in AD patients [46]. Brain regions such as hippocampus and cortex are highly sensitive to oxidative stress, mitochondrial dysfunction and neuroinflammation [47,48]. The expansive nature of oxidative damage in AD includes mitochondrial dysfunction, $A\beta$ formation, tau aggregation and alterations in calcium signaling [48]. All these changes lead to alterations in the transcriptional activity of various pathways, over-production of MDA, and reduced activity of superoxide dismutase, catalase, and GSH [47-49]. $A\beta_{(1-42)}$ is well known oligomer to causes oxidative stress, microglia and astrocytes activation and over expression of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . [50]. In the present study, $A\beta_{(1-42)}$ infused brain tissues exhibit an increase in oxidative stress, pro-inflammatory cytokines and nitrite level

which stay in good agreement with its previous findings [51]. However, lupeol treatment significantly attenuated $A\beta_{(1-42)}$ induced oxidative-nitritive stress and decreased the levels of pro-inflammatory cytokines, confirming its antioxidant and anti-inflammatory properties [52]. Earlier reports have also suggested that oral administration of lupeol decreases the secretion of pro-inflammatory cytokines such as TNF- α , and IL-1 β and inhibit the over activation of microglia and astrocytes [52]. It has been reported that lupeol act through various mechanism and down-regulates various apoptotic molecules like Bax, cytochrome C and caspases that leads to neuronal death [52]. Moreover Lupeol has also been reported to exhibit neuroprotective action against the glutamate induced exitotoxicity in mouse hippocampal cells [22]. Thus, the observed anti-inflammatory antioxidant effects of lupeol help to decrease the neuronal oxidative stress and may offer novel strategies in the treatment of age-related AD.

Conclusion

In the current study, lupeol halt $A\beta_{(1-42)}$ induced behavioral, biochemical and neurochemical abnormalities in rats. Lupeol was able to improve behavioral changes, restore neurotransmitters levels, decreased oxidative-nitrosative stress and reduced inflammatory processes. Together these findings suggest multiple therapeutic approach of lupeol towards $A\beta_{(1-42)}$ induced dementia and suggest lupeol as a potential therapeutic molecule to treat AD like symptoms.

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Conflict of Interest

The authors declare no competing financial interest.

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