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Research Article

COGNITION ENHANCING AND NEUROPROTECTIVE EFFECT OF CARVACROL IN OKADIAC ACID INDUCED OXIDATIVE STRESS, INFLAMMATION AND NEURODEGENERATION IN MICE

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ABSTRACT

Alzheimer's disease is a progressive, multifactoral, debilitating neurodegenerative disorder. The major hallmarks include extracellular plaques composed of β-amyloid, intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, inflammation, degeneration, synaptic dysfunction and oxidative stress. It is known that injecting Okadiac acid (OKA) into rodent brain induces hyperphosphorylation of tau at some of those sites that are found to be hyperphosphorylated in tau preparation obtained from AD patient brains. The aim of the study was to evaluate the protective effect of carvacrol at 2 doses (25 mg/kg i.p.and 50 mg/kg i.p). Carvacrol (cymophenol, or 5-isopropyl-2-methylphenol) is a monoterpenoid phenol present in the essential oils of medicinal and aromatic plants. Carvacrol was administered to swiss albino mice for 35 days. OKA was administered intracerebroventricularly on day 15. Memory was evaluated by employing morris water maze (MWM) test and recording the escape latency. Oxidative stress parameters, AChE level and TNF-α level was estimated in brain tissue homogenates. Carvacrol reduced the escape latency in the MWM test at both dosed. Administration of carvacrol significantly reduced oxidative stress and inflammation. It also significantly inhibited AChE. Thus it should be evaluated further for its prophylactic/ therapeutic potential.

KEYWORDS: *neuroprotective, neurodegeneration, Alzheimer's disease, neurons*

Introduction

The prevalence of Alzheimer's disease (AD), the most common cause of dementia worldwide, is continuously increasing due to ageing world population. AD accounts for up to 80% of all dementia [1]. The key pathological changes observed in the brain of a person suffering from AD are inflammation, plaques composed of Aβ (β amyloid), hyperphosphorylation of tau leading to formation of neurofibrillary tangles (NFTs), synaptic dysfunction and widespread neurodegeneration [2].

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According to the cholinergic hypothesis, there is dysfunction of acetylcholine containing neurons and this leads to cognitive impairment. This hypothesis has been the basis for employing AChE inhibitors such as Rivastigmine and Donepezil in the treatment of AD. Based on animal experimentation and postmortem and antemortem brain analysis, a number of cholinergic disruptions such as changes in choline transport, nicotinic and muscarinic receptor expression, acetylcholine release, neurotrophin support, and axonal transport may contribute to cognitive abnormalities in aging and AD. Cholinergic abnormalities may also contribute to the deposition of toxic neuritic plaques in AD [3]. Acetylcholinesterase inhibitors however provide only

symptomatic relief. The amyloid hypothesis has been by far the most accepted hypothesis and β-amyloid (Aβ) has been the main target of research for development of newer molecules. However, due to multiple failures such as the unsuccessful aggregated Aβ AN-1792 Active Immunization study [4,5] in 2001 and failures of various BACE inhibitors [6] and γ-secretase inhibitors [7,8] have put the credibility of the Amyloid hypothesis to question. According to the tau hypothesis, hyperphosphorylation of the microtubule associated protein (tau) leads to formation of intracellular neurofibrillary tangles (NFTs). Oligomeric forms and

tau filaments are released after neuronal death and they stimulate microglial cells and the cascade of neurodegeneration continues [9].

OKA is a selective protein phosphatases 1 (PP1) and protein phosphatase 2A (PP2A) inhibitor [10]. Inhibition of PP2A leads to hyperphosphorylation of tau protein (microtubule associated protein). In the brains of AD patients, a downregulation of PP2A has been observed. [11]. Thus drug design approach to upregulate PP2A might help reduce the pathological change brought about due to tau hyperphosphorylation. Kamat et al have shown that i.c.v administration of OKA into the rodent's brain causes cognitive impairment by due to hyperphosphorylation of tau and amyloid β deposition [12].

Carvacrol (cymophenol, or 5-isopropyl-2-methylphenol) is a monoterpenoid phenol present in the essential oils of medicinal and aromatic plants such as oregano (Origanum vulgare), wild bergamot (Citrus aurantium var. bergamia Loisel), pepperwort (Lepidium flavum), thyme (Thymus vulgaris and Thymus zygis), Spanish origanum (Thymbra capitata), summer savory (Satureja hortensis), white thyme (Thymus serpyllum) and winter savory (Satureja montana) [13,14]. In a study carried out to evaluate the effect of carvacrol in an animal model of chronic restraining stress, it was found that carvacrol ameliorated the oxidative stress parameters in the brain, liver and kidney [15]. Carvacrol has been shown to possess strong antioxidant properties and thus it may be effective in preventing and inhibiting several diseases [16]. Aydın et al have demonstrated that carvacrol increased the total antioxidant capacity of cultured primary rat neurons [17]. Carvacrol posses strong free radical scavenging activity. Due to this property, it significantly enhances glutathione levels and enhances radical scavenging capacity [18]. Carvacrol demonstrated significant hepatoprotective and hypolipidemic effect against D-GalN induced hepatotoxicity in rats. This effect was partly attributed to its capacity to curb oxidative stress [19,20]. Carvacrol has been found to significantly down-regulate the expressions of mRNA and protein expressions of TNF-α, IL-6, iNOS, COX-2 and NF-κB in hepatotoxic rats thus showcasing anti-inflammatory activity [21]. Carvacrol activates the peroxisome proliferator-activated receptors (PPAR) α and γ [22]. PPARγ represents an attractive therapeutic target for the treatment of AD [23]. Carvacrol attenuates diabetes associated cognitive defects in rats [24]. Carvacrol has been shown to possess neuroprotective effects against methotrexate induced toxicity in rats by decreasing the pro-inflammatory response [25]. In traditional Iranian Medicine, carvacrol containing plants have been reported to have improving cognitive abilities[26]. Carvacrol possesses cognition enhancing effect in the behavioural parameters evaluated in scopolamine and Aβ induced dementia in rats [27], however the biochemical and histopathological effects have not been explored. It has been observed that AChE inhibitory effect exerted by carvacrol is 10 times stronger than that exerted by its isomer thymol, although thymol and carvacrol have a very similar structure [28]. Thus even a slight modification in structure can bring about a substantial amplification in its pharmacological effect. Thus due to its acetylcholine esterase inhibitory potential and its anti-oxidant and anti-inflammatory properties, we decided to evaluate the neuroprotective and nootropic efficacy of carvacrol in Okadiac acid (OKA) induced memory impairment in mice.

Materials and methods

Animals

Swiss albino mice (25-35g) were used for the study. The study protocol was approved by the Institutional Animal Ethics Committee of Institute of Chemical Technology, Matunga, Mumbai (ICT/ IAEC/ 2017/ P 04) and all experiments were carried out in accordance with the guidelines laid down by Committee for Control and Supervision of Experimentation on Animals (CPCSEA). Animals were procured from National Institute of Biosciences, Pune and housed in polypropylene cages for 7 days for acclimatization before starting the experiment protocol. The animals were housed in a 12:12 hour light:dark cycle and were provided standard pellet feed and purified water ad libitum.

Experimental design and intraceribroventricular (i.c.v.) administration of OKA

Mice were divided into four groups (N=8). Drug/ saline dosing was carried out for 35 days and animals were sacrificed on the 36th day. The animals in the sham control group received saline i.p. daily and saline i.c.v on day 15. The disease control group was administered saline i.p. daily and OKA (200ng i.c.v) on day 15. Carvacrol was administered at 2 dose levels. One group received 25 mg/kg i.p. and the other group received 50 mg/kg i.p. OKA was administered i.c.v on day 15 by performing stereotaxic surgery. Mice were anesthetized with a combination of ketamine (100mg/kg i.p.) and xylazine (10mg/kg i.p.) and placed in the stereotaxic frame (Steolting Co., Illinois USA). The scalp was incised with a scalpel and the skull was exposed. The lateral ventricle was located (coordinates from bregma: anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −2 mm) and a small hole was drilled for the insertion of the cannula. The Hamilton micro syringe was positioned in the cannula and a solution of OKA in normal saline (200ng/4 µl) was injected slowly. The cannula was held in position and removed slowly to avoid backflow. Post surgical care included administration of painkiller and antibiotic injections.

Behavioral analysis

Morris water maze (MWM) test

The effect of carvacrol on learning and spatial memory was determined using the Morris water maze test. It has been shown to be a highly sensitive test for assessing damage to the hippocampus [29-31]. The apparatus consisted of a circular pool of 122 cm diameter, 50 cm height and was filled to a depth of 30 cm with fluid (milk powder was dissolved in water to make it opaque). A fixed platform (10 ×10 cm2) was placed 1 cm below the surface of water so that it would be hidden and the animal would have to remember the location of the platform based on spatial clues to escape from the fluid pool. Brightly colored spatial clues were placed surrounding the perimeter of the pool to help the mice navigate the way to the hidden platform. The pool was divided into four equal quadrants and the hidden platform was placed in the third quadrant. The location of the platform and the surrounding clues was maintained uniform throughout the training phases and also during the evaluation. Mice were randomly placed in one of the other three quadrants with their face towards the wall of the pool during the training phase. They were allowed to swim find the hidden platform in120s. If the mouse failed to do so, he was guided towards the platform and was allowed to stay on the platform for 30s in order to explore the spatial clues. Training was carried out consecutively on four days with a 30 min interval between training sessions. Escape latency. The amount of time taken by the mouse to locate the hidden platform (Escape latency in seconds) was evaluated on day 33.

Biochemical evaluation

Tissue processing for biochemical studies

The animals were euthanized by CO2 overdose on day 36. Perfusion was carried out with ice-cold 0.1 M phosphate buffer (pH 7.4) and the brain was isolated and weighed. 10% tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 4000 rpm for 10 min. The supernatant was separated and stored at -80° C for carrying out biochemical evaluation.

Acetylcholinesterase inhibitory activity

AChE activity correlates with cholinergic function. The method described by Ellman et al., 1961was employed to study AChEi function[32]. 40 µl of the tissue homogenate supernatant was mixed with 2.6 ml of 0.1M phosphate buffer and 100µl of DTNB (39.6 mg DTNB and 15 mg of NaHCO3 dissolved in 10 mL of 0.1 M phosphate buffer). 20 µl of 75 mM acetylthiocholine iodide was added and the change in absorbance was monitored per minute for 5 minutes. Acetylcholinesterase activity was calculated using formula;[33]

R= 5.74×10-4×A/ CO

where,

R-Rate in moles of substrate hydrolyzed/min/g of brain tissue

A-Change in absorbance/min.

CO-Original concentration of the tissue (mg/ml).

Oxidative stress parameters

Lipid peroxidation-MDA content

Lipid peroxidation is a process in which which oxidants attack lipids such as polyunsaturated fatty acids (PUFAs) that contain C-C double bond [34]. Malondialdehyde (MDA) is formed as a secondary product during lipid peroxidation. The content of MDA which is indicative of the extent of lipid peroxidation, was assayed in the supernatant in the form of Thiobarbituric acid reactive substance (TBARS) by the method described by Niehaus and Samuelsson, 1968. Thiobarbituric acid (TBA) reacts with MDA to produce a red couloured complex (peak absorbance of 535 nm). 0.1 ml of the supernatant and 2 ml of TBA-TCA-HCl (1 : 1 : 1) reagent (0.37% thiobarbituric acid, 0.25 N HCl, and 15% TCA) were heated on a boiling water bath for 15 min. After cooling, it was centrifuged at 4000 rpm for 10 min at room temperature. The absorbance of the supernatant was read at 535 nm using a spectrophotometer [35]. The MDA content was determined by using a standard curve.

Reduced glutathione (GSH) concentration

GSH, the most abundant low molecular weight thiol compound, plays a vital role in protecting cells from toxic substances and oxidative stress [36]. As per the method described by Moron et al., 1979, 0.4 ml of the supernatant was precipitated with an equal quantity of 20% TCA and centrifuged at 10,000 rpm at 4°C for 20 min. 0.25 ml of the supernatant thus obtained was then mixed with 2 ml of 0.6 mM DTNB reagent and the final volume was made up to 3ml with 0.2M phosphate buffer. DTNB reacts with GSH to form a yellow chromophore 5- thionitrobenzoic acid (TNB). The absorbance was read at 412 nm and the content was extrapolated from a standard curve[38].

Catalase activity

Catalase, the enzyme that breaks down H2O2 and also reacts with many other substrates, is the second most abundant enzymatic antioxidant that attenuates the levels of ROS that have been observed to rise during pathological conditions [38,39]. According to the method described by Aebi, 1984, change in absorbance at 240nm was indicative of decomposition of H2O2. 0.1 ml of the supernatant, 0.8 ml of phosphate buffer (50mM, pH 7.0) and 0.1 ml of 0.02% Triton X-100 were mixed and incubated at for 10 min at room temperature. This mixture was mixed with 2.0 ml of 0.03 M H2O2 and the change in absorbance was monitored 240nm for five minutes by recording the absorbance every minute. The activity of catalase was expressed as micromole of H2O2 decomposed/ min/ mg protein [40].

Superoxide dismutase (SOD) activity

SODs form the front line of defense against ROS mediated injuries in the body [41]. Natural SOD levels are known to drop due to aging [42]. This makes one more susceptible to oxidative stress related disorders as the body ages. SOD activity was measured by the method described by Marklund and Marklund, 1974. This method is based on the. 10 µl of tissue homogenate, 180 µl of phosphate buffer and 10 µl of pyrogallol were added to each well of a 96-well microplate. The ability of SOD to prevent the auto-oxidation of pyrogallol is the principle of this method. The absorbance was read at 325 nm for 5 min. 50% inhibition of autoxidation of pyrogallol was considered as one unit activity of SOD [43].

Proinflammatory cytokine (TNF-α)

TNF-alpha content in the brain was estimated sing commercially available ELISA kit (Krishgen biosystems). Results are expressed as pg/mg protein.

Results:

Morris water maze (MWM) test

The time taken by the mice to find the hidden platform on day 33 was significantly increased in the disease control group (as compared to vehicle control) and the escape latency was reduced significantly after administration of both doses of Carvacrol.

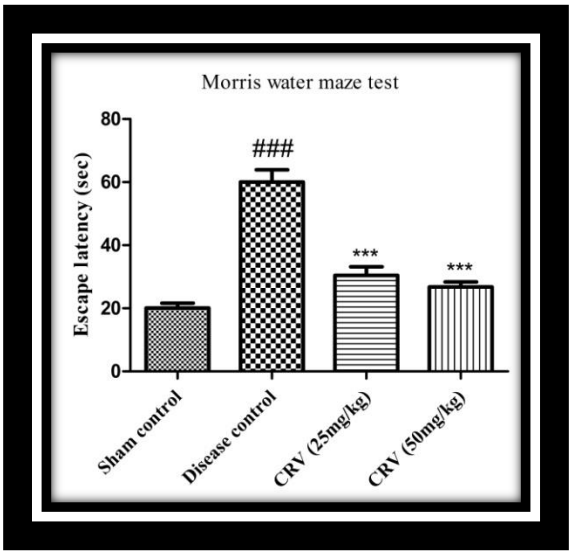

Figure 1: Escape latency (time taken by the animals to find the hidden platform). Values are expressed as Mean±SEM. ***P<0.001 as compared with disease control, ### P<0.001 as compared with vehicle control

Acetylcholinesterase activity

The activity of acetylcholinesterase was significantly increased after administration of OKA. A significant dose dependant inhibition was observed in its activity after treatment with carvacrol.

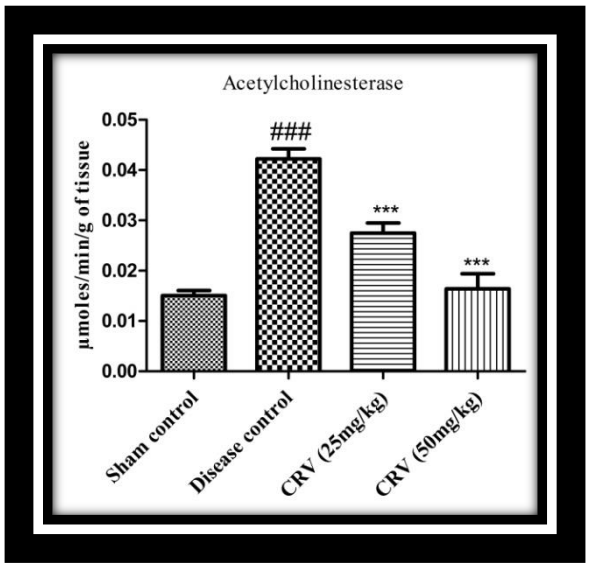

Figure 2: Acetylcholinesterase activity. Values are expressed as Mean±SEM. ***P<0.001 as compared with disease control, ### P<0.001 as compared with vehicle control

Lipid peroxidation-MDA content

The MDA content in the brain of carvacrol treated mice was significantly lower on both dose groups.

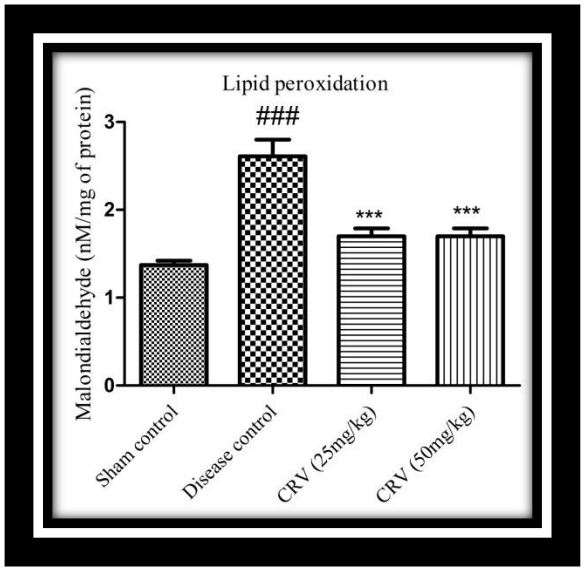

Figure 3: Lipid peroxidation. Values are expressed as Mean±SEM. ***P<0.001 as compared with disease control, ### P<0.001 as compared with vehicle control

Reduced glutathione (GSH) concentration

The GSH content in the brain of the disease control group was significantly lower. Treatment was carvacrol lead to a significant increase in the brain GSH concentration at both doses.

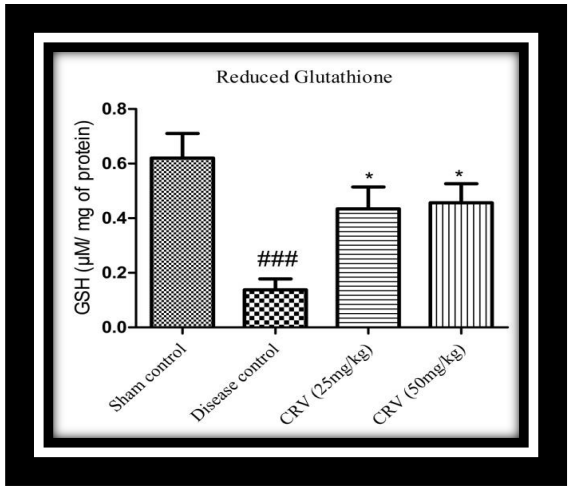

Figure 4: GSH content. Values are expressed as Mean±SEM. *P<0.05 as compared with disease control, ### P<0.001 as compared pared with vehicle control

Catalase activity

The reduction in catalase activity due to OKA administration was significantly reversed by carvacrol administration at both dose levels.

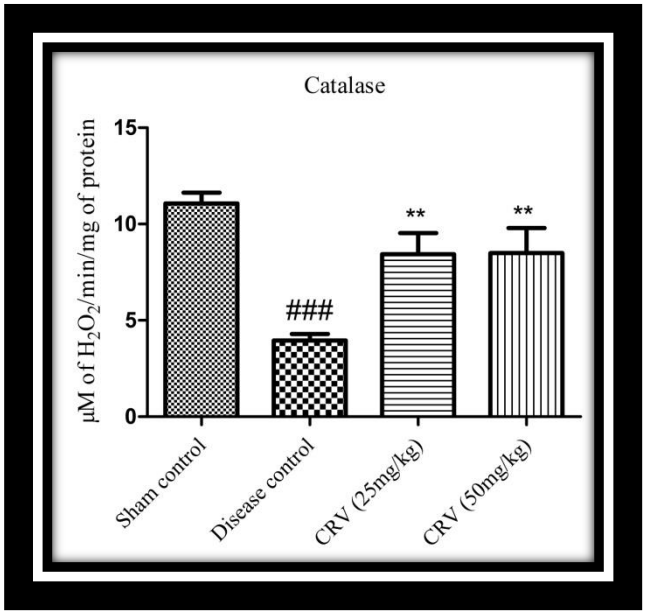

Figure 5: Catalase activity. Values are expressed as Mean±SEM. **P<0.01 as compared with disease control, ### P<0.001 as compared pared with vehicle control

Superoxide dismutase (SOD) activity

The reduced SOD activity was significantly revitalized dose dependently by carvacrol treatment.

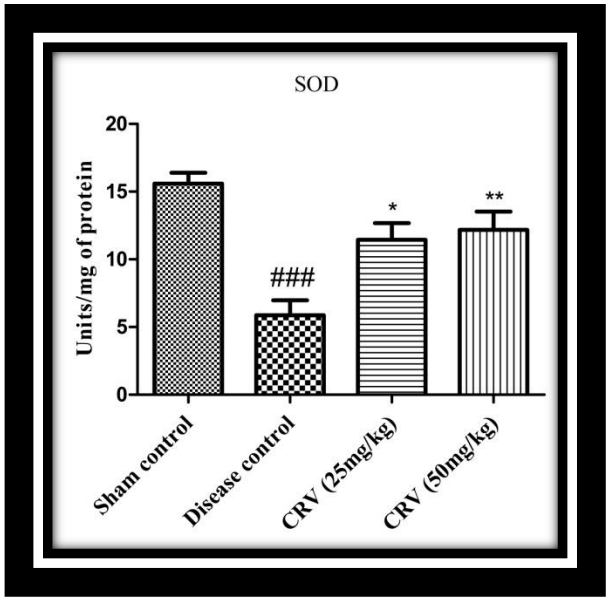

Figure 6: SOD activity. Values are expressed as Mean±SEM. **P<0.01, *P<0.05 as compared with disease control, ### P<0.001 as compared pared with vehicle control

TNF-α

There was a significant increase in the proinflammatory marker TNF-α in the brain after OKA was administered. This increase was significantly reversed in a dose dependant manor.

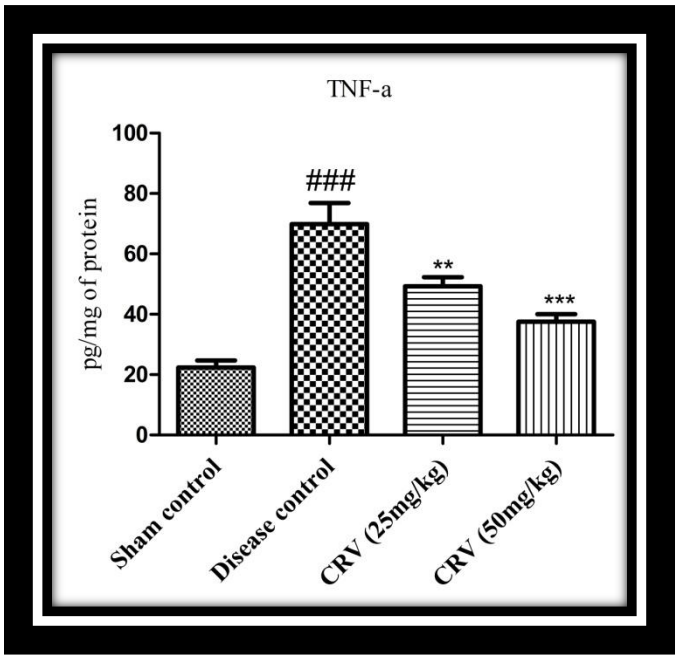

Figure 7: TNF α activity. Values are expressed as Mean±SEM. ***P<0.001, **P<0.01 as compared with disease control, ### P<0.001 as compared pared with vehicle control

Discussion

It has been proven that OKA administered by a single local injection or by chronic i.c.v infusion into rodent brain induces hyperphosphorylation of tau at some of those sites that are found to be hyperphosphorylated in tau preparation obtained from AD patient brains [44]. Thus, we decided to administer OKA i.c.v. to study the effect of carvacrol on the changes in memory, oxidative stress, inflammation and increase in cholinergic activity. The model has been previously standardized in our lab. Oxidative stress occurs when the balance between the generation of free radicals and their detoxification by the biological system is disturbed. The excessive free radicals thus generated lead to oxidative damage of tissues by reacting with proteins, lipids, nucleic acids. This disrupts the normal functioning of the cells and leads to diseases [45,46]. Carvacrol significantly reduced the escape latency in MWM test, thus indicating improvement in learning and memory. OKA administration lead to a significant increase in proinflammatory cytokine TNF-α and oxidative stress parameters. There was also a significant increase in acetylcholineserase activity. Both doses of carvacrol significantly reduced neuroinflammation marker TNF-α and caused a reduction in oxidative stress. There was a significant reduction in MDA level and an increase in GSH content. Activity of SOD and catalase was also significantly increased. The increase in acetylcholinesterase activity was also significantly reversed by carvacrol administration.

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