

Research Article

Pharmacological Investigation of Carvacrol Against D-galactose-Induced Cognitive Memory Impairment

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Abstract

Aging is a physiological process involving senescence that declines the normal body functions and adaptability of the body to metabolic and environmental stress. The role of inflammatory mediators is widely studied in cognitive functions in the past but the efficacy of dietary supplements against brain senescence is not well established. In the current study, we determined the neuroprotective potential of carvacrol against D-galactose-induced cognitive impairment. Male mice 25-30 grams were used to determine the anti-aging potential of carvacrol and we also demonstrated the role of p-NF-κB in the aging process. We observed the increased expression of p-NF-κB further upregulated TNF-α and COX-2 expression. Moreover, we observed marked lipid peroxidation along with a significant reduction in levels of antioxidant markers that lead to senescence in the cortical brain region (decreased percentage of intact neurons). Carvacrol has significantly reversed these detrimental effects caused by D-galactose by declining the expression of p-NF-κB. Our findings suggest that carvacrol has attenuated D-galactose-induced cognitive impairment by inhibiting p-NF-κB.

Keywords: Memory, cognition, carvacrol, neuroprotection, antioxidant, inflammation.

Introduction

Aging is a physiological process that eventually targets the cognitive functions of the brain. The decline in cognitive functions makes humans prone to various neurodegenerative disorders. Among these neurodegenerative disorders, Alzheimer's disease is the most prevalent. Other neurodegenerative diseases associated with aging include Parkinsonian disease and Huntington's disease (Yanker et al., 2008). Aging plays a central role in the development of multiple brain dysfunctioning and morbidity and there is a need to explore the molecular basis involved in aging. According to recent studies, some pathological alterations in the temporal brain region anticipate dementia in the geriatric population and there is altered brain activation (Hedden and Gabrieli,

2004). Extensive research is being done on the possible treatment of cognitive deficits associated with aging, still, there is room for studies because no possible treatment strategy is developed to date. Several herbal extracts are being used to support cognitive brain functions but no supporting data to answer their efficacy to prevent cognitive decline (Zhang et al., 2016). Moreover, poor understanding of the prognosis and development of cognitive impairment in aging hinders the treatment strategies. Therefore, there is a need to understand the pathogenesis involved in cognitive suppression in aging so that new treatment strategies can be developed.

It is evident from the literature that IKK/NF-κB signaling pathway has a substantial role in aging.

This pathway is activated by oxidative stress, genotoxic elements, and inflammatory mediators, upregulation of several cytokines, cell senescence, and inflammation. The transcription of p-NF- κ B was found to significantly increase in aging cells (Tilstra et al., 2011). A genetically modified group of mice in which the NF- κ B gene was inhibited has shown a marked reduction in senescence-related to aging, which supports the evidence that the NF- κ B gene could be a plausible target (Adler et al., 2007). Moreover, activation of NF- κ B is associated with the upregulation of certain inflammatory mediators including IL-6, IL-8, IL-7, MIP-3, and ICAM leading to anti-growth and enhanced pro-inflammatory phenotype, which worsen the aging process. Moreover, the oxidative stress upregulates the expression of NF- κ B, and activated NF- κ B is associated with the upregulation of many pro-inflammatory genes and apoptotic markers that causes inflammation and apoptosis in the prefrontal region of the cortex and hippocampus including TNF- α , COX-2, p-JNK, and caspase-3 (Naeem et al., 2021). Therefore, IKK/NF- κ B signaling pathway should be considered for the development of newer therapies for aging-related cognitive loss.

It is evident from the literature that oxidative stress leads to mitochondrial dysfunction. Mitochondrial damage is widely associated with cognitive deficits associated with aging-related neurodegenerative disorders such as Alzheimer's disease (Wallace et al., 2005). Mitochondrial damage can lead to the production of free radicals. Moreover, mitochondrial DNA damage associated with Alzheimer's disease and aging leads to the overproduction of reactive oxygen species (ROS) (Coskun et al., 2004). In addition to the generation of superoxide and hydrogen peroxide, the availability of redox-active iron is a major determinant of ROS-mediated cellular damage. Elevated levels of redox-active iron accumulate in the normal aging brain and several neurodegenerative diseases (Zecca et al., 2004). This mitochondrial damage further upregulates

the IKK/NF- κ B pathway, therefore, worsening the aging process.

D-galactose (D-Galac)-injected rodent models recapitulate many features of brain aging and have been extensively applied to study the mechanisms of brain aging (Budni et al., 2016). D-Galac has been considered a senescent model for age-related neurodegenerative disease. It induces oxidative stress which triggers memory impairment, neuroinflammation, and neurodegeneration (Ali et al., 2015). D-Galac is a reducing monosaccharide and if systemically exposed, causes accelerated senescence in several organs and is widely being used as an ideal agent to induce brain aging in animal models (Wei et al., 2005).

Natural drug substances are getting fame and are considered a useful source in drug development due to their potent antioxidant action. Several drugs are reported for their antioxidant activity against a variety of mediators involved in inflammation and free radicals (Uddin et al., 2020). Carvacrol is chemically a mono-terpenoid called 5-isopropyl-2-methyl phenol). It is obtained from essential oils from the plants of the Labiatae family such as thyme and oregano (Nagoor et al., 2017). Carvacrol has been reported for its neuroprotective properties against LPS-induced neurodegeneration whereas carvacrol has shown a marked reduction of p-NF- κ B and lipid peroxidation and upregulated the activity of catalase, GST, and GSH (Naeem et al., 2021).

Materials & Methods

Chemicals

Carvacrol (W224502), D-Galac (314062-47-0), glutathione (GSH), trichloroacetic acid (TCA), 1-Chloro-2,4-dinitrobenzene (CDNB), N-(1-Naphthyl) ethylenediamine dihydrochloride and 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies such as anti-TNF- α (SC-52B83), mouse monoclonal anti-p-NF κ B (SC-271908), anti-COX-2 antibody (sc-1746), and ABC

Elite kit, and 3, 3'-diaminobenzidine peroxidase (DAB) were purchased from Santa Cruz Biotechnology USA. The horseradish peroxidase-conjugated secondary antibody was obtained from Abcam UK (ab-6789). ELISA kits for p-NF-kB (Cat # SUB28069) were procured from Shanghai Yuchun Biotechnology (China), and COX-2 (cat. No. E-EL-H5574) and TNF- α (cat. No. E-EL-R0019) ELISA kits were purchased from Elabscience.

Animals

Male C57BL/6N mice weighing 25-30 g were housed three per cage under a 12-hour light/dark cycle with free access to water and food at the animal house of Riphah Institute of Pharmaceutical Sciences (RIPS) under standard laboratory protocols (temperature: 22 \pm 1 $^{\circ}$ C; humidity: 50% \pm 10%). All experimental procedures were carried out according to the guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996), approved by the RIPS Ethical Committee (Reference No: REC/RIPS/2017/010).

Experimental Protocol

Male C57BL/6N mice were classified into three groups (I) Control (C), mice were treated with normal saline and 5% DMSO as a vehicle for two months, (II) D- galactose group (D- galac) mice were treated with D- galactose for two months at 100mg/kg, (III) D- galactose (100mg/kg) was administered i.p. for two months and carvacrol (25mg/kg) was administered for 1 month (D-galac+Car). Carvacrol was dissolved in 5% DMSO and the final volume was made with normal saline. Both vehicle and carvacrol were administered i.p. 12 hours before evening for one month.

Behavioral Assessment

Morris Water Maze Test (MWM)

MWM was performed according to the procedure described earlier (Ali et al., 2015). A clear platform about 10 cm in diameter and 20 cm in height was

concealed 1 cm below the surface of the water and settled in the middle of a quadrant. Mice (n=10/group) were trained for six days using a hidden object under the center of a specified quadrant. Latency time (time to find the hidden object) was evaluated for six days. On the seventh-day probe, the test was performed in which a hidden object was removed and mice were allowed to swim in the tank for 1 minute. The time spent by mice in the previously allocated quadrant was measured as memory consolidation after training. The mice were videotaped during training and probe testing.

Y-Maze Test (YMT)

We performed the Y-Maze test as previously described (Imran et al., 2020). Special wooden Y-shaped apparatus was used of specified dimensions (length 50 cm, width 10 cm, and 20 cm height). Each mouse was placed in the center of the maze and videotaped for eight minutes. The consecutive entries of mice into three arms were defined as spontaneous alternation behavior. Whereas percentage alteration behavior was calculated as:

Alteration behavior (%)=[(successive triplet entries \times entries into 3 different arms successively)/(total number of entries)-2] \times 100

Social Interaction Test (SIT)

Subject mice were placed in their respective cage and two empty cages were placed adjacent to the home cage. A female C57BL/6N mouse was placed in one of the empty cages and the mice were videotaped for the next three minutes (Poon et al., 2012).

Light Dark Box Test (LDB)

The test was performed as mentioned previously (Khan et al., 2016). The light-dark box apparatus comprises two boxes called a light box and a dark box. Each mouse was evaluated for the time spent in the light and dark box.

Elevated Plus-Maze Test (EPM)

EPM was performed as described (Naeem et al., 2021). Mice were placed in the center of the maze facing an open arm and videotaped for five minutes. Each mouse was evaluated for the time spent in the open arm.

Histological Preparation

After performing, behavioral analysis mice were decapitated and brain tissues were extracted. A sharp blade was used to cut 3mm thick sections of the brain which were preserved in 4% formalin solution. Tissues were then fixed in paraffin and then four micrometer thin coronal sections were cut using a microtome. Later nissl staining and immunohistochemistry were performed.

Cresyl Violet Staining

Staining was performed as described (Tripathy et al., 2013). For rehydration different concentrations of ethanol were used for 1 min in decreasing order as 100%; 90%, 70%; 50%. After rehydration slides were washed with distilled water for 15 sec. Slides were then stained for 4 minutes using Cresyl violet stain and later washed again with distilled water for 15 sec. Dehydration was then performed using 50%; 70%, 90%, and 100% ethanol solution for 60 sec. The later clearing was done using xylene and mounting was aided by DPX mountant. Later light microscope was used to take images and saved them in TIFF format and analysis was done using ImageJ software.

Immunohistochemistry

Immunohistochemistry was done as previously performed by Zulfiqar et al., 2020. Slides were deparaffinized using xylene and then rehydrated using increasing concentrations of ethanol (100%; 90%; 80% and 70%). For antigen retrieval slides were then processed by enzymatic method and washed with PBS for 5 min. Later slides were dipped in 3% H₂O₂ and washed with PBS. Normal goat serum was then applied and incubation was done for 2 hrs. The primary antibodies (COX-2, p-NF- κ B, and TNF- α) were

applied and slides were incubated for 24 hrs. Slides were then washed with PBS and coated with secondary antibodies then incubation was done for 2 hrs with an ABC kit in a humidified chamber. Staining was then performed using DAB; dehydration was the next step using increasing concentrations of ethanol (70%; 80%; 90% and 100%). The clearing was done using xylene and mounting was done by DPX mounting media. Microscopy was then used light microscope and analysis was performed by ImageJ software.

Antioxidant Analysis

Lipid Peroxidation (LPO)

Lipid peroxidation was performed as described by Malik et al., 2020. The cortex of each mouse was homogenized in 10 mL of 20mM Tris-HCL at pH 7.4 and 4°C and the supernatant was collected after centrifugation (1000g for 10 min 4°C). 40 microliters of freshly prepared ferric ammonium sulfate were added to the supernatant and incubation was done for 30 min at 37°C. Later 75 microliter of 0.8% freshly prepared solution of TBA was added to the mixture. At 532nm wavelength absorbance was measured using a plate reader.

GST, GSH & Catalase Activity

GST, GSH, and catalase activity were performed on collected supernatant as described previously (Iqbal et al., 2020; Ali et al., 2020; Al Kury et al., 2020) respectively. Results were expressed in terms of μ moles of conjugate/min/mg of protein.

ELISA

ELISA of TNF- α , COX-2, and p-NF- κ B was performed according to the manufacturer's instructions. Initially, the cortex was homogenized by using Silent Crusher M and centrifuged at 1000g for 10 min 4°C. The total concentration of protein was measured by a BCA kit from Elabscience. After performing the ELISA

procedure results were measured in terms of pg/mL.

Statistical Analysis

Data were represented as \pm SEM and analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post-hoc analysis by GraphPad Prism software. Data value greater than $p < 0.05$ was considered statistically significant. Results of immunohistochemistry and Cresyl violet staining were analyzed by ImageJ software. The symbol * indicates a significant difference relative to the saline group while # indicates a significant difference relative to the D-Galac group.

Results

Effects of Carvacrol on the D-Galac-Induced Behavioral Deficit

D-Galac-treated mice have shown a significant reduction in exploration as compared to saline in the EPM test and time spent in the open arm was also reduced (Figure. 1A and 1B, $**p < 0.01$ and

$***p < 0.001$). Similarly, D-Galac has shown a marked reduction in the number of entries and time spent in the lightbox in LDB (Figure. 1C and 1D, $**p < 0.01$ and $***p < 0.001$). In the MWM test D-Galac lead to a significant increase in escape latency (Figure. 1E, $***p < 0.001$) while in YMT alteration % was reduced in D-Galac treated mice in comparison to saline-treated mice (Figure. 1F, $***p < 0.001$). Carvacrol reverses the D-Galac-induced behavioral deficits as obvious from the EPM test, the number of entries and time spent in the open arm is markedly increased as compared to D-Galac group (Figure. 1A $##p < 0.01$ and $###p < 0.001$). A similar kind of result was obtained from the LDB test that is the number of entries and time spent in the light box were improved as compared to D-Galac group (Figure. 1C and 1D, $##p < 0.01$ and $#p < 0.05$). D-Galac+Car group has shown a significant increase in escape latency, alteration %, and interaction duration with female mice in MWM, YMT, and SIT respectively (Figure, 1E, 1F, and 1G, $#p < 0.05$).

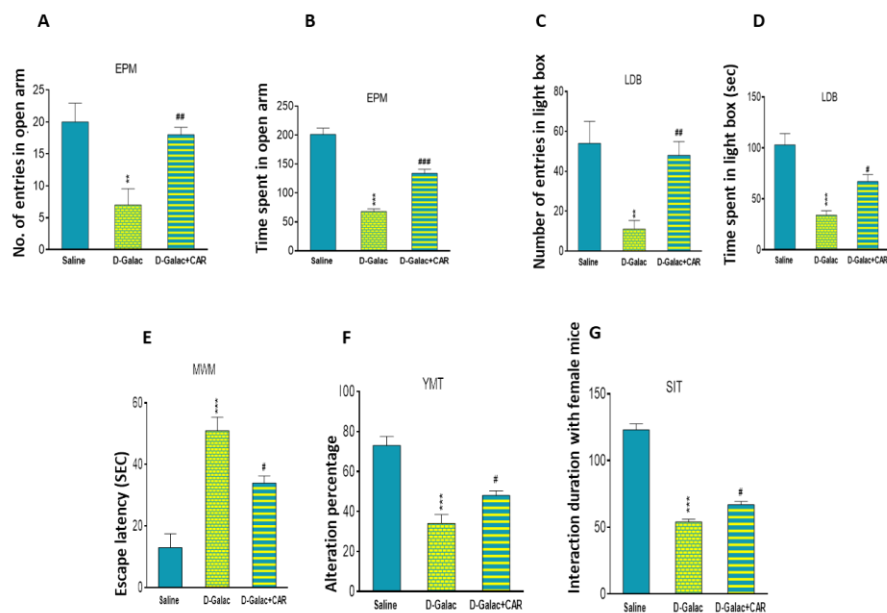


Figure 1. Effects of carvacrol on D-Galac induced behavioral deficits. Effect of CAR and D-Galac on EPM (A and B), LDB test (C and D), MWM test (E), YMT (F), and SIT (G). Data are expressed as mean \pm SEM and analyzed by one-way ANOVA followed by Bonferroni multiple comparison test using GraphPad Prism 6 software. Saline, D-Galac, and D-Galac+CAR groups were represented (n=10/group). $***p < 0.001$ and $p < 0.01$ indicates significant difference with saline group while $###p < 0.001$, $##p < 0.01$ and $#p < 0.05$ indicates significant difference with D-Galac group.

Effect of carvacrol on histopathological counting of intact neurons of cortex in Cresyl violet staining

The mice showed a significant reduction in neurons in the cortex in D-Galac (** $p < 0.001$) group in comparison with the saline group. The Saline group has shown 12% of contact neurons in the cortex while in D-Galac has shown 3% of intact

neurons. Carvacrol has significantly reversed the effect of D-Galac on intact neurons in Cresyl violet staining. In the D-Galac+CAR group, the percentage of intact neurons has improved up to 6% ($\#p < 0.05$) in comparison with D-Galac group alone.

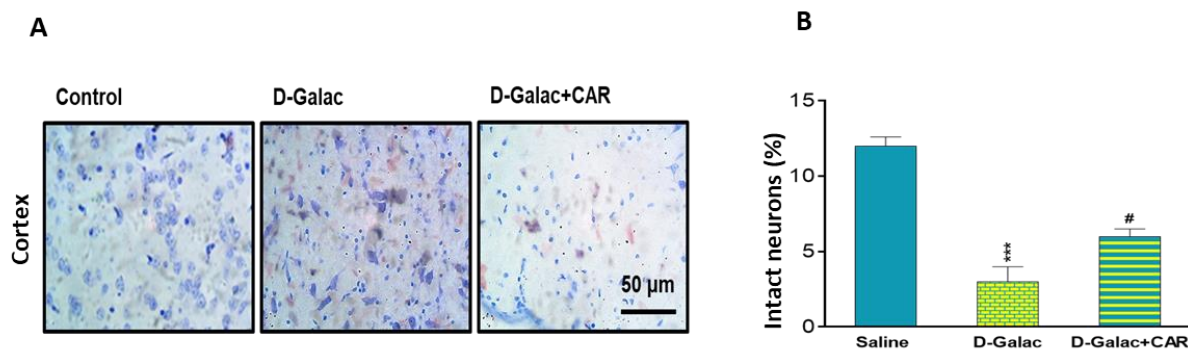


Figure 2. Effect of carvacrol on histopathological counting of intact neurons of cortex in Cresyl violet staining. (A) Scale bar 50 μm , magnification 40X. Photomicrographs obtained from saline, D-Galac, and D-Galac+CAR are shown. (B) Data are expressed as mean \pm SEM (Intact Neuron %). ** $p < 0.001$ indicates a significant difference compared with the saline group while $\#p < 0.05$ indicates a significant difference compared with D-Galac group.

Effects of Carvacrol on D-Galac-Induced Neuro-inflammation

It is evident from the literature that there is a substantial contribution of neuroinflammatory mediators in cognitive memory impairment; therefore we measured the effects of carvacrol on these mediators. We studied the levels of expression of p-NF- κB , TNF- α , and COX-2 in the cortex by ELISA and for further validation, we performed an immunohistochemical analysis of these mediators in the cortex. The levels of expression of p-NF- κB both in ELISA and immunohistochemistry were upregulated by D-Galac group (Figure. 3A and 3B,

** $p < 0.01$ and ** $p < 0.001$, respectively). Similarly, the levels of expression of TNF- α both in ELISA and immunohistochemistry were increased significantly by D-Galac group (Figure. 3C and 3D, ** $p < 0.01$). Moreover, the expression of COX-2 was also enhanced in ELISA and immunohistochemistry of the cortex by D-Galac group (Figure. 3D and 3E, ** $p < 0.001$ and ** $p < 0.01$, respectively). While carvacrol has significantly reversed the effects of D-Galac group, there is a significant reduction in the expression levels of p-NF- κB , TNF- α , and COX-2 as evident from the results (Figure. 3).

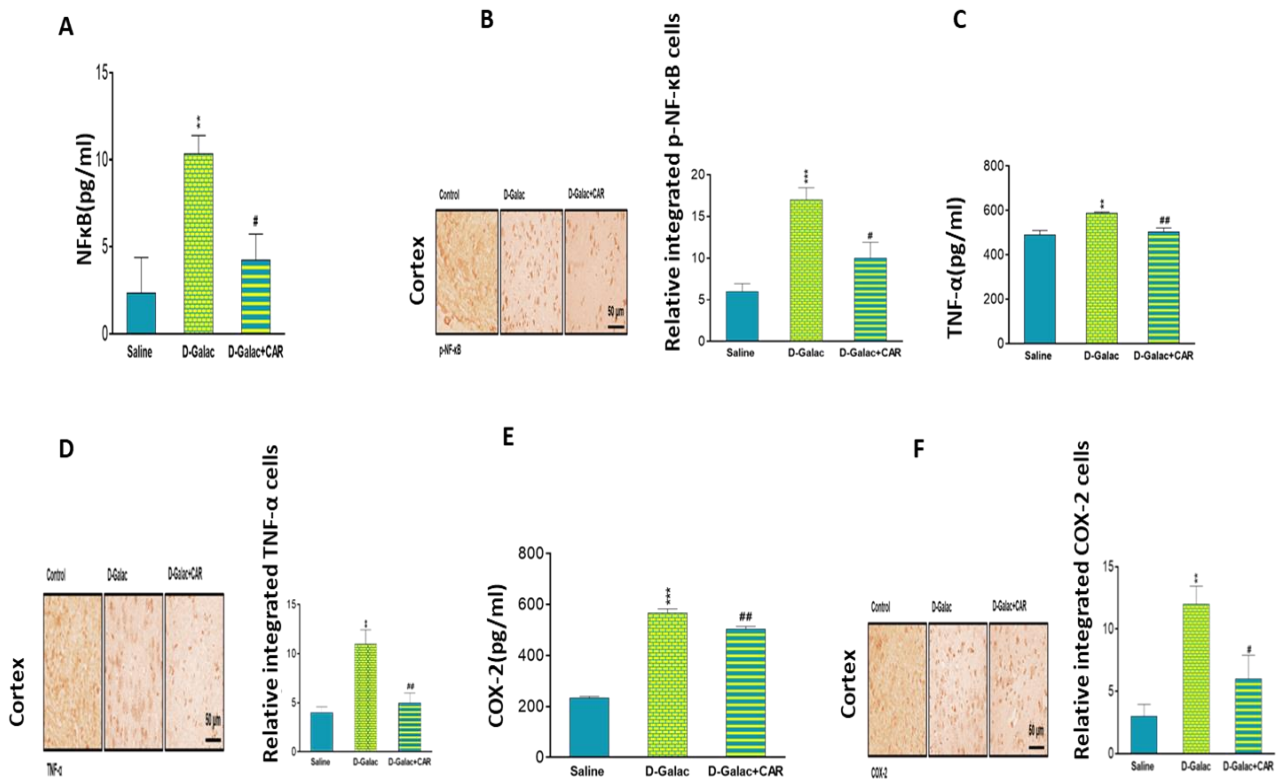


Figure 3. Effects of carvacrol on D-Galac-induced neuroinflammation. (A) p-NF-κB levels were measured by ELISA, (B) Immunohistochemistry results of p-NF-κB in the cortex, (C) TNF-α levels were measured by ELISA, (D) Immunohistochemistry results of TNF-α in the cortex, (E) COX-2 levels were measured by ELISA, (F) Immunohistochemistry results of COX-2 in the cortex. Scale bar 50 μm, and magnification 40X. Data are expressed as mean ± SEM. ***p<0.001 and **p<0.01 indicates the significant difference compared with the saline group. ##p<0.01 and #p<0.05 indicates a difference compared with the D-Galac group.

Effects of Carvacrol on D-Galac-Induced Lipid Peroxidation

The neuroprotective effect of carvacrol against D-Galac-induced oxidative damage and lipid peroxidation was determined by performing lipid peroxidation activity in the cortex of male C57BL/6N mice. Lipid peroxidation was

measured in terms of thiobarbituric acid reactive species (TBARS). The levels of TBARS were significantly upregulated by D-Galac group (Figure 4A. ***p<0.001), whereas carvacrol has prominently reversed the effects of D-Galac alone (Figure 4A. #p<0.05).

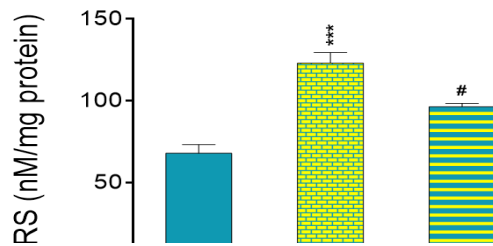


Figure 4. Effect of carvacrol on D-Galac-induced lipid peroxidation. Effect of D-Galac and carvacrol on TBARS. Data are expressed mean \pm SEM. *** p <0.001 indicates results compared with saline and # p <0.05 indicates results compared with D-Galac group.

Effects of Carvacrol on D-Galac-Induced Oxidative Stress

To determine the antioxidant potential of carvacrol against D-Galac-induced oxidative stress in the cortex of male C57BL/6N mice we performed the catalase, GSH, and GST assay. The levels of catalase, GST, and GSH are

significantly reduced by the administration of D-Galac (Figure. 5A, B, and C, ** p <0.01), whereas carvacrol has upregulated the levels of catalase, GST, and GSH (Figure. 5A, B and C, # p <0.05).

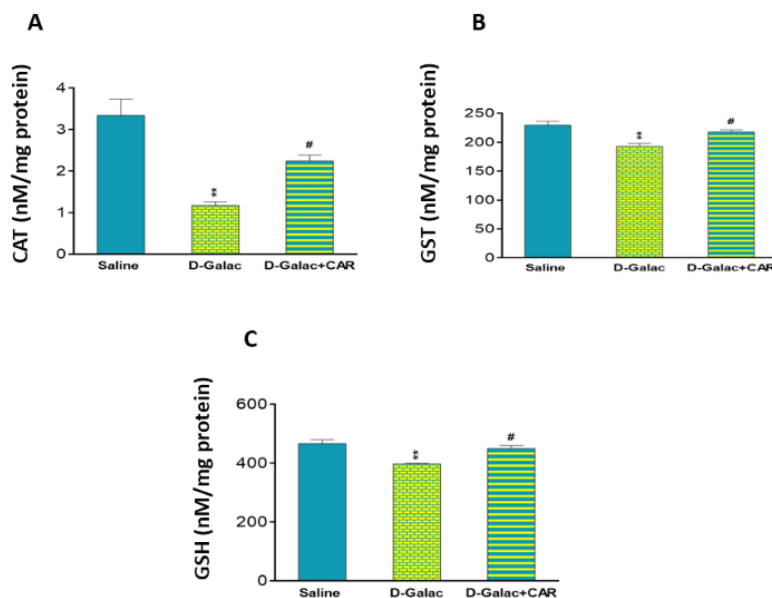


Figure 5. Effects of carvacrol on D-Galac induced oxidative stress. Effects of carvacrol and D-Galac on CAT (A), GST (B), and GSH (C). Data are expressed as mean \pm SEM. ** p <0.01 indicates the results compared with the saline group and # p <0.05 indicates results compared with D-Galac group.

Discussion

In the current study, we investigated the protective effects of carvacrol against aging in animal models possibly by attenuating the oxidative and inflammatory cascade as carvacrol exhibited anti-inflammatory potential in the previous studies (Guimar et al., 2012; Silva et al., 2013). Natural drugs are particularly investigated for their beneficial pharmacological potential against various diseases not only to delineate the underlying pathology but to develop newer and safer therapeutic alternatives.

D-Galac-mediated neurological deficits were promptly overcome by the administration of carvacrol, as evident from the behavioral analysis. Carvacrol has shown significant improvement by showing prominent escape latency in the MWM test and has significantly ameliorated alteration % in YMT and has shown a prominent change in the duration of interaction with female mice in SIT. Carvacrol has also shown similarity in EPM and LDB results with previous research in which carvacrol has attenuated LPS-induced neurodegeneration (Naeem et al., 2021).

Several studies investigated the involvement of reactive oxygen species in the pathophysiology of various neurodegenerative disorders (Liu et al., 2017; Uddin et al., 2020). Similarly, oxidative stress can limit the brain's capacity to combat the damage-causing anxiety-like symptoms. We have demonstrated a significant reduction in lipid peroxidation by carvacrol in comparison with D-Galac. Improved expression of catalase, GSH, and GST was also seen by treatment with carvacrol. Our findings suggest that carvacrol is a potent antioxidant therefore, it has the potential to combat neurodegeneration and improve the symptoms associated with cognitive deficits such as anxiety of aging or anxiety associated with aging. Our results of Cresyl violet staining indicate that carvacrol has significantly increased the number of intact neurons suggesting the neuroprotective potential of carvacrol (Yu et al., 2012).

The prefrontal cortex and the hippocampus have been implicated in several types of memory and behavior, according to various research. Our results demonstrated motor function deficits as well as decreased locomotor and exploratory behavior following chronic D-Galac exposure similar to previous studies, which were alleviated by carvacrol therapy, showing carvacrol's neuroprotective capacity. Furthermore, free radicals, oxidative stress, and depletion of antioxidant defenses have been linked to D-Galac-induced neurotoxicity. Because of the high levels of unsaturated lipids and the high rate of oxidative metabolism in brain tissues, ROS generation leads to lipid peroxidation, which alters cellular structures and causes protein and DNA damage as well as apoptosis and cell death. As a result, brain tissues are particularly vulnerable to oxidative damage. Our results confirmed these findings and showed a decrease in CAT, GSH, and GST levels, as well as an increase in LPO levels. Treatment with carvacrol increased the levels of these antioxidant enzymes considerably while decreasing LPO levels, which might be linked to its neuroprotective properties. These findings support prior research that found carvacrol causes a considerable increase in antioxidant enzyme activity and a decrease in LPO levels in the stroke and epilepsy model. Therefore, carvacrol might be involved in the removal of oxygen or its precursors, suppression of ROS, or activation of the endogenous antioxidant defense system, which are all plausible mechanisms. In addition, the histological results corroborated these findings, as neuronal damage caused by D-Galac poisoning was revealed, and tissue damage was reversed following treatment with carvacrol. It is widely recognized that COX-2, NF- κ B, and TNF- α constitute the cornerstone of neuronal damage in several neurodegenerative disorders, including D-Galac neurotoxicity. Our results supported earlier findings and indicated an increased level of pro-inflammatory and inflammatory mediators, which were diminished by carvacrol therapy.

Conclusions

Our *in vivo* results indicate that carvacrol has shown potent antioxidant potential and it has anti-neuroinflammatory properties that mediate the neuroprotection against D-Galac-induced cognitive memory impairment in the aging model. Moreover, our results indicate that carvacrol has inhibitory properties against p-NF- κ B (mediator of many pro-inflammatory genes including TNF- α and COX-2). Therefore, carvacrol has anti-neuroinflammatory potential and it may offer new therapeutic options to prevent and manage neuroinflammation in neurodegenerative disorders such as aging.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

There was no specific funding available for this project.

Study Approval

Yes. The study was approved by the Institutional Review Board & Ethics Committee of the Riphah Institute of Pharmaceutical Sciences.

Consent Forms

NA.

Authors Contribution

FAS conceptualized the study and wrote the final manuscript, SR helped in the analysis and writing the first draft, did the experimental analysis, and FAS supervised the whole project and wrote the final manuscript.

Acknowledgments

The authors thank the Dear of Riphah Institute of Pharmaceutical Sciences for helping conduct this study.

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