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ORIGINAL ARTICLE

Does Carvacrol Have Useful Effects on Multiple Sclerosis Treatment?

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Abstract

Introduction: Multiple sclerosis (MS) is the second cause to disabling young people in the world. Attempts to find an appropriate cure for MS have not been successful yet. In recent years, trying to use plants to cure illness has been increased. Carvacrol from Labiatae family may give us the opportunity to deal against MS.

Material and Method: 24 female Lewis rats (180–200 g) were used for the experimental autoimmune encephalomyelitis (EAE) study and were randomly assigned to three groups (n=8 per group): Control, EAE, and Carvacrol-treated EAE. An additional eight female Lewis rats were used for the biochemical toxicity assessment of Carvacrol (n=4 per group: Control and Carvacrol). The EAE model was induced using guinea pig spinal cord antigen injected at the base of the tail. Pertussis toxin was administered intraperitoneally to facilitate blood–brain barrier disruption. Biochemical parameters were measured to evaluate the toxicity of Carvacrol, and Real-Time PCR was performed to assess the expression of GFAP, CCL5, CXCL1, OPN, and β2-adrenergic receptor genes.

Result: Toxicity tests showed no significant differences in Carvacrol group compare to Control group. The results of RT-PCR were unexpected. In case of CCL5, CXCL1, OPN and B2-Adrenergic, there was no significant change. However, in our study, the EAE-model could increase the expression of GFAP ($P<0.05$) which shows the ability of this model to induce MS-like condition. On the other hand, Carvacrol could diminish the expression of all genes significantly ($P<0.05$) in comparison to Control group.

Conclusion: From this study and our previous studies, it could be impressed that Carvacrol with antioxidant, anti-inflammatory etc. properties is worth of much investigations related to MS.

Keywords: Carvacrol, EAE-model, PCR, Real-Time, Multiple sclerosis.

Introduction

The youth health in all countries over the world can be suffered from different causes. The second place of disabling disorders for young people is for Multiple sclerosis (MS) which can even paralyze the person (1). The number of individuals sufferings from MS is increasing annually. In Iran, statistics show that at least 120/000 people are in challenge with MS (http://www.iranms.ir). Although the exact cause and mechanism for MS have not been agreed, there are some suggestions in this field. For instance, many researchers have claimed that genetic profile and environmental factors such as harmful sun emissions, vitamin D deficiency and smoking can affect the process of the MS (2). Two main events in MS are inflammation and neurodegeneration (3) which come together in progressive stages of the disease. In the beginning of the disease process, Oligodendrocytes are often influenced more than other brain cells. These cells are the source of myelin production and the result of destruction of their activity will be myelin disappearing in plaque regions (4). Inflammatory plaques can spread in parts of central nervous system and include immune cells and immunoglobulins in which have entered into the brain after breaking of blood brain barrier (BBB) (5). Attempts on finding a proper cure for MS has been focused on remyelination and neuroprotection properties of agents. In these strategies, some improvements have taken place. for example, Biotin and Clemastine can improve myelin regeneration and Simvastatin and Ibudilast can protect neurons from degeneration (6). However, despite these progressions, multiple sclerosis claims victims yet. In recent years, herbal medicine has attracted attentions worldwide and attempts are towards the nature to cure illnesses. There are various reasons for this movement. One of commonest reasons is the price of chemical drugs and their accessibility. In other hand, unwilling short and long-term side effects of these drugs have convinced scientists to explore new ways of cure. Nowadays, the potential of plants to treat different disease is not ignorable and this is because of their antioxidant, anti-inflammatory, antimicrobial, etc. properties (7). Ginkgo biloba, Curcuma longa, Hypericum perforatum, and others are good examples of medicinal plants used for MS treatment (8). It has been a while (more than 5 years, perhaps) that our group has started to find the effect of Carvacrol on MS condition (9, 10). This compound is obtained from plants Labiatae family members (such as Satureja, Thymbra and Thymus) and its ability to manage some situations like oxidation, sepsis, inflammation, diabetes, etc. has been proven (11). Carvacrol is a liquid phenolic monoterpenoid with effective neuroprotective impact useful for Epilepsy and Parkinson disease treatment (9). Up to now, there is no dangerous side effects reported in literature for own Carvacrol itself. However unlimited using of plants containing this substance can make allergic reactions, increased iron absorption and digestion problems which cannot relate them to Carvacrol without comprehensive explorations (12). In the previous investigations, we showed that Carvacrol can alter gene expression in the central nervous system as well as body weight and MS clinical scores in rats(9, 10). So, we decided to measure the expression of some other inflammatory genes [GFAP (Glial Fibrillary Acidic Protein), B (Beta)2-Adrenergic, OPN (Osteopontin), CCL5 (CC-chemokine ligand 5), CXCL1 (C-X-C motif ligand 1)] related to MS disease to achieve a better insight on Carvacrol's potential for MS treatment.

Material and Methods

Animals

The animals used in this study were adult female Lewis rats. Each group was randomly assigned, consisting of eight rats/group, under the following conditions (Table 1):

Table 1. Rat Conditions in Research Time

Groups	3 (Control, EAE, carvacrol-treated EAE)
Weight	180-200 g
Humidity	60%-70%
Temperature	22˚C ± 2˚C
Dark/light cycle	12 h
Food and water accessibility	Free
Ethics approval reference number	Lorestan University of Medical Sciences, Lorestan, Iran (IR.LUMS.REC.1396.309)

Abbreviations: EAE, experimental autoimmune encephalomyelitis.

Experimental Autoimmune Encephalomyelitis (EAE) Induction

We applied the experimental autoimmune encephalomyelitis (EAE) model of MS according to previous studies (13), because it best simulates the clinical features of MS. To induce this model, immunization was performed by injection of guinea pig spinal cord antigens (Pasteur Institute, Iran) dissolved in Complete Freund's Adjuvant (CFA, Millipore Sigma, US) and an equal amount of water (0.5 mL/rat, in tail base) along with pertussis toxin (Millipore Sigma, US) dissolved in distilled water (twice, two h and 48 h after immunization, 250 ng/rat each time, i.p.) (14). These steps were performed after the animals were anesthetized using a mixture of Ketamine and Xylazine (Millipore Sigma, US, 1:4 ratio, i.p.). After a few days, the first signs of disability were observed, and we separated the rats with the same features into special groups.

Biochemical Toxicity Test

A crucial issue in using medicinal plants is determining safe doses to avoid toxicity. Therefore, we checked laboratory potassium (K^+), sodium (Na^+), bilirubin (BIL and D), alkaline phosphatase (ALK), prothrombin test (PT), serum glutamic oxaloacetic transaminase (SGOT), creatinine (Cr), and blood urea nitrogen (BUN) concentrations to determine the possible toxic effects of carvacrol on vital parameters. To achieve this goal, eight female Lewis rats were divided into two groups: control and carvacrol (four rats/group, 180±20 g). Carvacrol was administered intraperitoneally for 21 consecutive days (25mg/kg). At the end of the experiment, blood samples (1 mL from each rat) were collected directly from the heart using syringes and stored in ethylenediaminetetraacetic acid-containing vials. Samples were centrifuged for 15 minutes at 3000 rpm, and finally, serum samples were harvested using a sampler.

Ribonucleic Acid (RNA) Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Our group performed this part according to the manufacturer's instructions and the following steps. Briefly, the lumbar spinal cord was the target for ribonucleic acid (RNA) isolation, and the TRIZOL (Sigma-Aldrich) technique was used. RNA purity and concentration were determined using a UV spectrophotometer (Pharmacia Biotech, Cambridge, UK). The YTA kit (Yekta Tajhiz Azma Co., Iran) was used to produce complementary DNA (cDNA). The enzyme activity was stopped by incubating all samples for 5 minutes at 70◦C. The genes GFAP, OPN, CCL5, CXCL1, and β2-Adrenergic (Table 2) were assessed, and GAPDH was used as a housekeeping gene. Primers were designed and checked using NCBI and BLAST. Finally, we used triplicate experiments and the $2^{-\Delta\Delta Ct}$ method to confirm gene expression (15).

Table 2. Primer Sequences Used for Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Gene	Forward (5′ to 3′)	Reverse (5′→3′)
β2-Adrenergic	GTTGTGCGTCACAGCCAGCA	AGATACGATAGAGGAAGCGA
CXCL1	GGCAGGGATTCACTTCAAGA	GCCATCGGTGCAATCTATCT
CCL5	CGTGAAGGAGTATTTTTACACCAGC	CTTGAACCCACTTCTTCTCTGGG
OPN	AACGGATGACTTTAAGCAAGAAAC	TACTGTTCATCAGAAACAGGGAAA
GFAP	TGGCCACCAGTAACATGCAA	CAGTTGGCGGCGATAGTCAT

Study Design

Immunization to induce the model was performed on the first day of the experiment. In addition to immunization, pertussis toxin was injected (intraperitoneally) in two steps, once at 2 h and again at 48 h after immunization. On day 12, we began intraperitoneal injections of carvacrol (25 mg/kg, Millipore Sigma, US) until day 29. From days 1 to 12, we assessed the body weight and the score of clinical signs of the animals, as reported in our previous studies (9). Finally, on day 29, the animals were sacrificed, and spinal cord samples were extracted to determine the effect of carvacrol on the targeted genes (Figure 1).

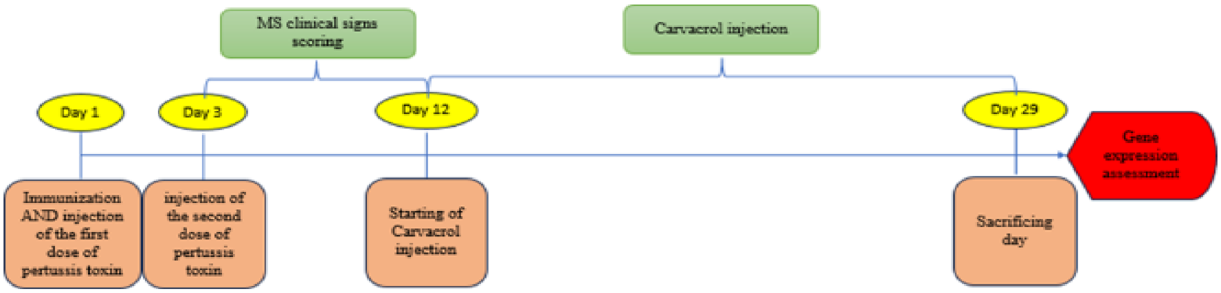

Figure 1. Study design for whole research process

Statistical Analysis

Data were analyzed using GraphPad Prism 9 software (Inc., La Jolla, US). Tukey's post hoc test was used to evaluate the differences between the groups. The results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was set at *P*-values<0.05.

Results

Effect of Carvacrol on Biochemical Factors

Briefly, as shown (Figure 2), evaluation of K^+, Na^+, BILT, and D, Alk, PT, SGOT, Cr, and BUN values after 21-day administration of carvacrol showed changes in the measured parameters; however, these changes were not statistically significant compared to the control group (Table 3). Therefore, carvacrol is safe for use in experimental studies.

None of the parameters showed significant changes.

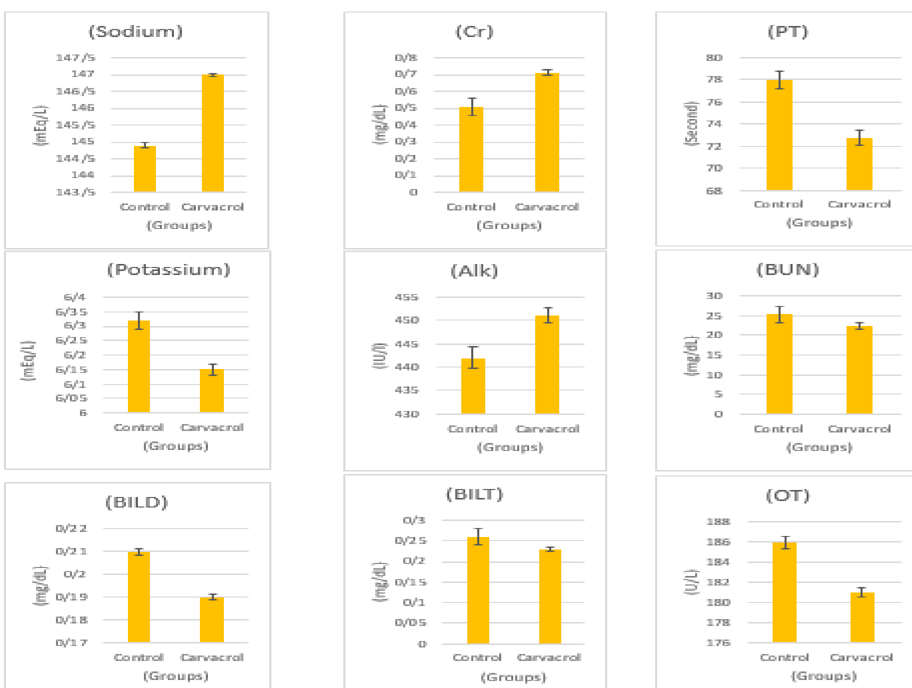

Figure 2. Result of toxicity assessment of Carvacrol.

Table 3. Quantitative Values of Vital Biochemical Parameter

		K^+	Na^+	BILD	BILT	Alk	PT	OT	Cr	BUN
1	Control group	6.32 ±1.33	144.9 ±2.43	0.21 ± 0.1	0.26 ± 0.019	442 ± 12.69	78 ± 15.25	186 ± 19.2	0.51 ± 0.18	25.35 ± 8.97
2	Carvacrol group	6.15 ± 0.8	147 ± 0.001	0.19 ± 0.001	0.23 ± 0.002	451 ± 9.93	72.75 ± 10.53	181 ± 10.2	0.71 ± 0.001	22.25 ± 2.5
3	*P*-value	0.840*	0.182*	0.134*	0.821*	0.178*	0.111*	0.121*	0.821*	0.547*

* Not significant.
Abbreviations: K^+, potassium; Na^+, sodium; BILT, total bilirubin; BILD, direct bilirubin; Alk, alkaline phosphatase; PT, prothrombin test; OT, serum glutamic oxaloacetic transaminase; Cr, creatinine; BUN, blood urea nitrogen.

Effect of Carvacrol on Expression of Inflammatory Genes (Glial Fibrillary Acidic Protein [GFAP], β2-Adrenergic, Osteopontin [OPN], CC-chemokine Ligand 5 [CCL5], C-X-C Motif Ligand 1 [CXCL1])

As shown in Figure 3, RT-PCR performed in this study showed that EAE induction in female Lewis rats altered the expression of the assessed genes compared to the control group. However, in most genes, these changes were not significant, whereas GFAP expression was significantly increased ($P<0.05$). Carvacrol used in this study decreased the expression of all the mentioned genes; compared to the control group, all reductions were significant ($P<0.05$).

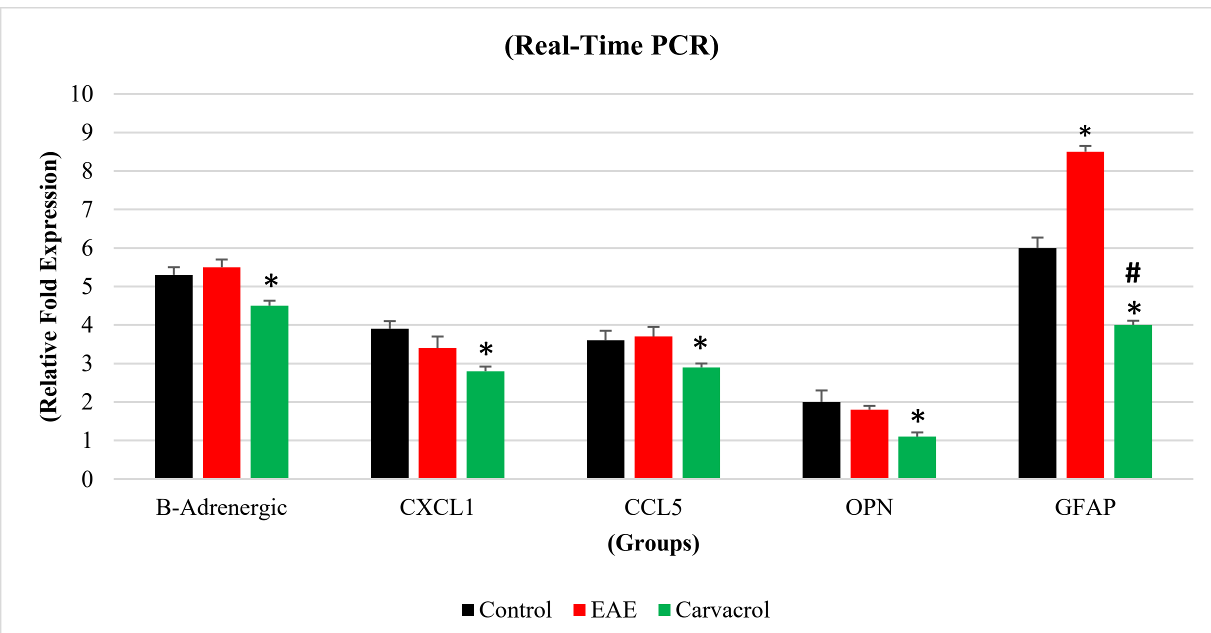

Figure 3. Comparison of β2-Adrenergic, C-X-C motif ligand 1 (CXCL1), CC-chemokine ligand 5 (CCL5), Osteopontin (OPN), and Glial Fibrillary Acidic Protein (GFAP) Gene Expression.

In most of the genes investigated in this study, no significant changes were seen. Data are expressed as mean ± SEM.
* $P<0.05$ v.s. Control group, # $P<0.05$ v.s. EAE group.

Discussion

In this study, EAE induction in the animal model was confirmed by scoring (9). The two main types of MS are relapsing-remitting MS (RRMS) and primary progressive MS (PPMS). The first is more common in women, and the second is more common in men (16). One of the main markers in almost all disease-related to the brain and neurodegeneration is inflammation. In this study, we targeted inflammatory genes to examine the effect of carvacrol on MS-like conditions (EAE model). GFAP (an astrocyte marker) has been related to MS in many studies. As Shaygannejad et al. showed in a systematic review in 2024 (17), most studies on MS have observed increased GFAP levels. Consistent with other studies, our results showed a significant increase in GFAP expression in the EAE group ($P<0.05$). However, carvacrol counteracted this effect and diminished the GFAP expression. The past decade has been a challenging period for investigating MS cures. Recently, trials have shown that CXCL1, which recruits immune cells (especially neutrophils), can be a promising marker for MS diagnosis. Therefore, researchers have focused on measuring its concentration in cerebrospinal fluid and plasma samples (18). Apparently, the conclusion about the CXCL1 in MS is an increased amount of it. However, these reports are controversial because no shift in CXCL1 have been observed in patients with MS in some studies (19). Indeed, we did not observe a significant change in CXCL1 expression in the EAE group. This is probably because CXCL1 levels are increased in the early stages of MS (19). Despite these facts, carvacrol used in this study significantly decreased CXCL1 compared to the control group ($P<0.05$). Another gene assessed in this study was CCL5, which promotes the migration of leukocytes towards damaged or inflamed tissues. By reviewing previous studies, it can be concluded that CCL5 may play a crucial role in attracting inflammatory cells to the brain during the acute phase of MS (20). In addition to CXCL1, no remarkable changes in CCL5 were observed in our study. This lack of change is due to the fact that we assessed the gene expression in the late phase of the disease, and because of the effect of carvacrol, this expression has been suppressed. If we could measure CCL5 protein levels, there would probably be changes. However, carvacrol resulted in a significant reduction in CCL5 compared to the control group ($P<0.05$). A group of researchers conducted a meta-analysis to examine the variation in OPN concentration in MS. At the end of the article, they included that MS is associated with overproduction of OPN (21), which acts as a proinflammatory cytokine involved in immune responses and tissue repair. However, as we observed for CXCL1 and CCL5, there was little change in OPN expression in the EAE-rat model of MS, and in contrast, carvacrol could affect the mentioned factor significantly compared to the control group ($P<0.05$). β2-Adrenergic signaling plays a crucial role in preventing MS by regulating glycogenolysis and reducing inflammatory factors. Therefore, metabolic and calcium balance regulation occurs through β2-adrenergic signaling (22). In our study, the EAE model did not significantly alter β2-adrenergic expression. However, carvacrol caused a significant change ($P<0.05$). This result was unexpected based on previous studies (22). The possible explanations for these results are diverse. For example, the age of animals, course duration, the dose of substances used for EAE induction, and the ability of pertussis toxin to breach the BBB may alter the results of our study compared to other studies.

Conclusion

As mentioned earlier, our group has been working on carvacrol for a long time. Unlike our previous studies, the results of the present study did not show significant differences for most of the evaluated genes. For GFAP, the result was substantial, which is crucial because it shows that the EAE model of MS can activate defensive reactions. In case of other genes, we may not have chosen the proper time, or other environmental conditions may have affected the experiment. Finding a new cure requires an extensive search, and for carvacrol, we are at the beginning, and more studies are needed.

Conflict of Interest The authors declared no conflicts of interest.

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