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ORIGINAL ARTICLE

The effect of carvacrol on respiratory syncytial virus infection in mice model: caution in the use of herbal medicines

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ABSTRACT

Background and Objectives: Respiratory syncytial virus (RSV) is one of the most common viruses associated with acute lower respiratory tract infections in infants, young children, and the elderly. Due to a lack of effective anti-viral drugs or vaccines, using an immunomodulatory strategy is probably the best option to decrease the burden of RSV disease. Here, we studied carvacrol as a known immunomodulator on RSV infection outcome in a mice model.

Materials and Methods: Balb/c mice were infected by intranasal inoculation of RSV-A2, and treatment started daily 24 h after infection. Mice were sacrificed on day five after infection and experimental analyses were performed to study airway immune cell influx, CD4 and CD8 subtypes, cytokine/chemokine secretion, lung histopathology, and viral load.

Results: Results showed that using carvacrol enhanced immune cell influx, cytokine/chemokine production, and virus titer, and aggravated lung pathology. Our result showed that carvacrol administration increased viral titer compared to the RSV-PBS group. Also, carvacrol significantly induced IFN-γ production and did not induce IL-10 production. Besides, carvacrol non-significantly increased lymphocytes and monocytes count but did not affect the neutrophil count.

Conclusion: Carvacrol at the concentration of 80 (mg/kg) did not show immunomodulatory activity to alleviate the RSV infection outcome. Further research is needed to uncover the effects of the carvacrol intervention on virus replication and immune responses following RSV infection. Many herbal remedies in use contain carvacrol. However, the use of herbal remedies to treat viral respiratory infections such as RSV has to be performed with caution.

Keywords: Human respiratory syncytial virus; Carvacrol; Immunomodulation; Interferon-gamma; Interleukin-10

INTRODUCTION

Respiratory syncytial virus (RSV) is the most common cause of respiratory illness in infants, young children and, the elderly, worldwide (1). Disease symptoms vary from a mild upper respiratory tract infection to severe bronchiolitis and pneumonia requiring hospitalization (2). The virus accounts for about 50% to 90% of hospitalizations for bronchiolitis and up to 40% of pneumonia (3). RSV disease mortality rates in children aged <5 years is estimated to be 200,000, with 99% occurring in developing countries (4). There is growing evidence that early life severe RSV infection may lead to recurrent wheezing and the development of asthma in later life (5). Due to a lack of vaccines, an effective treatment strategy is needed to decrease the burden of disease and its considerable long-term effects (6).

Although strategies that solely targeted the virus (anti-viral drugs) are obvious candidates to treat

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RSV illnesses, they did not meet their expectations and remain in infancy (7). The lung pathology developed in RSV-infected individuals is characterized by strong pulmonary inflammation, which is known as immunopathology (8). Inflammation in the upper and lower airways of infants with acute bronchiolitis is dominated by an intense immune cell influx, and this is accompanied by pro-inflammatory and anti-inflammatory chemokine/cytokine production and disease severity (2). There is, however, increasing evidence to suggest that targeting of RSV-associated immune response can alter virus replication, inflammation, and a pathology leading to improved disease outcomes (7, 9).

There are many options as immunomodulatory compounds, and the tendency to use herbal compounds will increase due to their low price and high efficacy (10). Carvacrol, as an herbal compound found in many plants of the *Lamiaceae* family, has immunomodulatory and anti-inflammatory action. It also has many other biological effects such as anti-obesity, anti-fungal, anti-viral, anti-bacterial, neuroprotective, cardioprotective, and anti-oxidant properties (11). Although the mechanisms of immunomodulatory and anti-inflammatory action of carvacrol are not clear completely (12), it has been discovered that carvacrol reduces the recruitment of immune cells, especially neutrophils (13). Furthermore, it has been shown that it increases IL-10 production and the ratio of IFN-γ/IL-4 as well as Th1/Th2 (12, 14). Here, we studied carvacrol as an herbal immunomodulator on RSV infection outcome in a mice model.

MATERIALS AND METHODS

Twenty-four female Balb/c mice (6-7 weeks old, weighing 17-20 g) were purchased from the Institute Pasteur of Iran (Karaj, Iran). Animals were kept in-house for one week before the experiment in the animal house of Tehran University of Medical Sciences with access to standard laboratory food and water *ad libitum*. This study was approved by the animal ethics committee of the Tehran University of Medical Sciences (No. 1398.047). Carvacrol was obtained from Sigma-Aldrich with a purity of 98% and diluted with normal saline serum as recommended. Virus stock was prepared as described previously by our groups (15).

Mice were randomly assigned into four groups: PBS-PBS, PBS-CAR, RSV-PBS, and RSV-CAR. According to the mentioned groups, mice were anesthetized with ketamine/xylazine and intranasally infected with RSV-A2 at 7.8×10^6 pfu/50 λ/mice dose. The carvacrol was injected i.p. 24 h after viral infection once a day at a dose of 80 (mg/kg). The control groups received an equal volume of phosphate-buffered saline (PBS). Animals were sacrificed by a lethal dose of ketamine (i.p.) on day five after infection, and bronchoalveolar lavage fluid (BALF) and lungs were obtained to measure airway immune cells influx, $CD4^+$, and $CD8^+$ T-cell populations, cytokine/chemokine secretion, lung histopathology, and viral load.

The lung was lavaged through a catheter inserted into the trachea and by flushing with ice-cold PBS, according to our previous work (15). The obtained BALFs were centrifuged, and the total number of cells was counted with Neubauer chambers. Differential leukocyte counts were performed on smears stained with Giemsa dye using standard morphological criteria. The amount of IFN-γ and IL-10 cytokines were determined in the BALF supernatants by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems and Invitrogen, respectively).

The $CD4^+$, and $CD8^+$ T-cell population in BALF was measured with the flow cytometry method. The specific volume of each sample that consisted of 2×10^5 cells was washed twice with FACS buffer (2% BSA in PBS) and incubated with fluorochrome-conjugated anti-CD3, anti-CD4 and anti-CD8 immunoglobulins (BD bioscience, USA) for 45 min at 4°C in the dark. The cells were washed twice with FACS wash buffer and analyzed with a flow cytometer (Life technologies, USA). FlowJo® software (Tree Star, Inc., Ashland, OR, USA) was used to analyze the data.

Histology slides were prepared as described previously by our group (15). Prepared slides were evaluated by light microscope, and lung pathology, peribronchial and perivascular infiltration in the lungs were scored as described previously (16). The average of the sum of each reading was compared among groups.

Total RNA was extracted from supernatants of BALF using viral high pure nucleic acid extraction kit following manufacturers' instructions (Roche, Germany). Real-time RT-PCR was carried out on an ABI PRISM 7900 sequence-detection system (Applied Biosystems, USA) using TagMan PCR Master Mix (Primer Design, UK), and results were expressed as copy number/ml as described previously (15).

RESULTS

Our results showed that the carvacrol increased viral titer compared to the RSV-PBS group (Fig. 1). RSV infection stimulates an inflammatory response in the airways comprising cytokines production and, a mixed population of immune cell infiltration, resulting in lung pathology. Compared with the control group, carvacrol significantly induced IFN- γ production (p-value = 0.024) and did not induce IL-10 production (Fig. 2). In this experiment, the level of immune cell infiltration was enhanced following carvacrol treatment in RSV-infected mice (RSV-CAR group), although it was not statistically significant compared to the RSV-PBS group (Fig. 3). The differential analysis of the leukocytes in the BALF of mice showed that carvacrol administration non-significantly increased lymphocytes and monocytes count, but did not affect neutrophil count (Fig. 4). Carvacrol treatment increased $CD4^+$, and $CD8^+$ lymphocytes count, but CD4/CD8 ratio non-significantly decreased following carvacrol treatment in RSV-infected mice (RSV-CAR group) (Fig. 5). According to the immune responses and viral titer, lung pathology scores were higher in mice of the RSV-CAR group than the RSV-PBS members (Fig. 6). In this experiment, exposure of mice to the PBS or carvacrol alone did not result in differences within the control groups.

DISCUSSION

The immunomodulatory activity of carvacrol has been well investigated in several studies (11, 17). However, the results of this study showed that carvacrol not only did not alleviate the RSV immunopathology but also worsen the infection outcome. Using

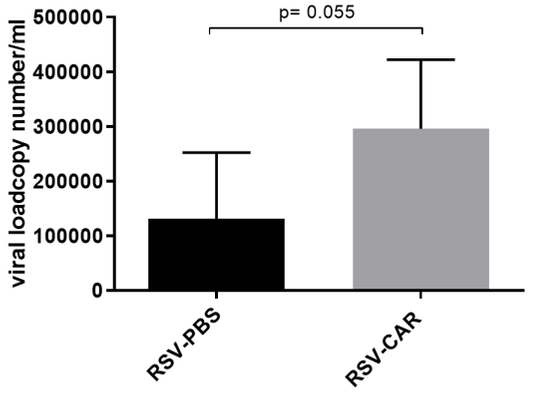

Fig. 1. The effect of carvacrol on viral titer following RSV infection: carvacrol insignificantly increased viral titer in RSV-CAR group compare to RSV-PBS group (p-value = 0.05).

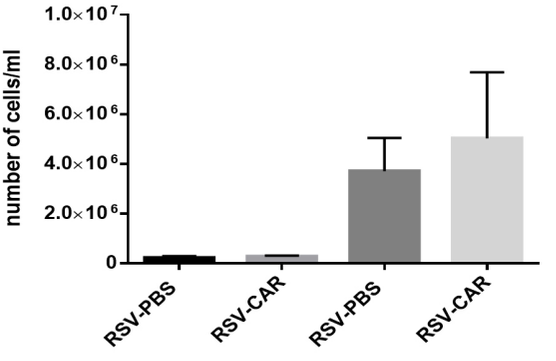

Fig. 3. The effect of carvacrol applying on the total number of WBC infiltration in BALF: the total number of WBC in RSV-CAR group was insignificantly higher than RSV-PBS group. Cells count was determined on day five after infection. Results represent the mean –SEM of six animals for each group.

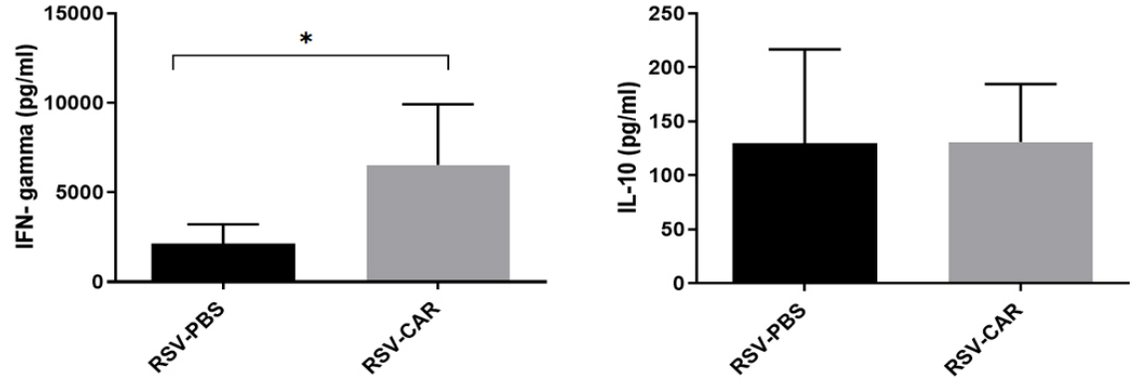

Fig. 2. The effect of carvacrol on cytokines levels: carvacrol induced IFN- γ production (p-value = 0.024) and did not affect IL-10 production. The measurement of cytokines was done after mice sacrificed on, the fifth day of infection by ELISA.

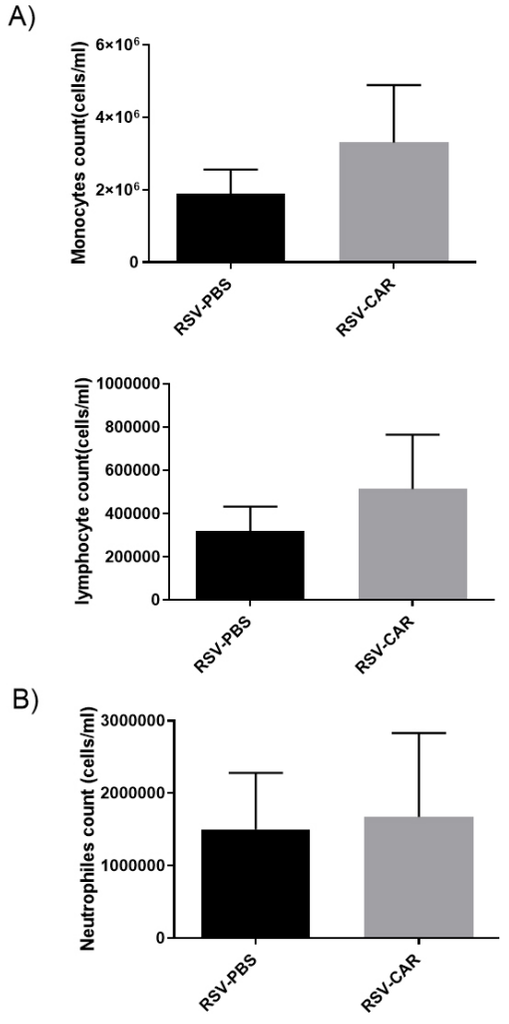

Fig. 4. Number of different leucocytes in BALF samples of mice in RSV-PBS and RSV-CAR groups: A) lymphocytes and monocytes count increased in RSV-CAR group compare to RSV-PBS group (p-value was not significant) B) there was not any difference in neutrophils count between two groups.

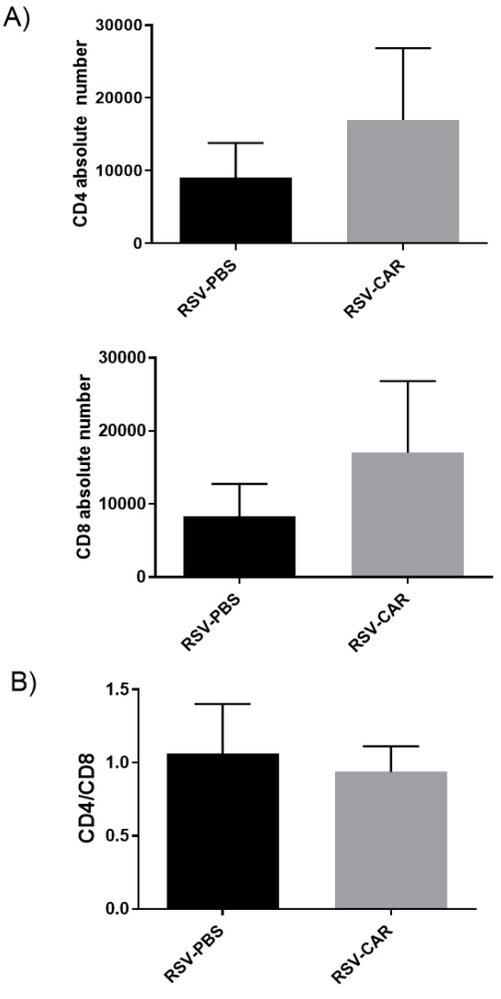

Fig. 5. A) Increase in the absolute number of CD4 and CD8 was observed in RSV-CAR members (p-value was not significant) B) the CD4/CD8 ratio slightly changed between RSV-PBS and RSV-CAR groups.

carvacrol enhanced the virus titer following by an increased cytokine/chemokine production and immune cells influx and ultimately aggravated lung pathology. The numbers of neutrophils did not change due to carvacrol administration, which was against our presumption that carvacrol could decrease the infiltration of neutrophils. Furthermore, flow cytometry results displayed an increase in CD8 and CD4 absolute number and a decrease in CD4/CD8 ratio, which indicates the exaggeration of immune responses with changing toward, Th1 direction. The higher score of histopathology of lung slides in the RSV-CAR group delighted the more severe infection resulting from of carvacrol administration.

The results were totally against our expectation, and we assumed some hypotheses to explain the promiscuous effect of carvacrol in the RSV mice model. It is evident that the concentration of agents is very critical in the experiment and can even distort the results. As an example, Rajabli et al. showed that carvacrol at the concentration of 1 mM increased NheA and HblC toxins in Bacillus cereus infection, but at the concentration of 2 mM and 8 mM inhibited their production (18). In Stojanovic et al. study, carvacrol at a concentration of 10 mg/kg caused a decrease in α-amylase and lipase activities that led to the amelioration of pancreatic damage in a rat model, but at a concentration of 50 and 100 (mg/kg) increased the activities of the enzymes, and exacerbated the condition (19). Although the administrated dose of carvac-

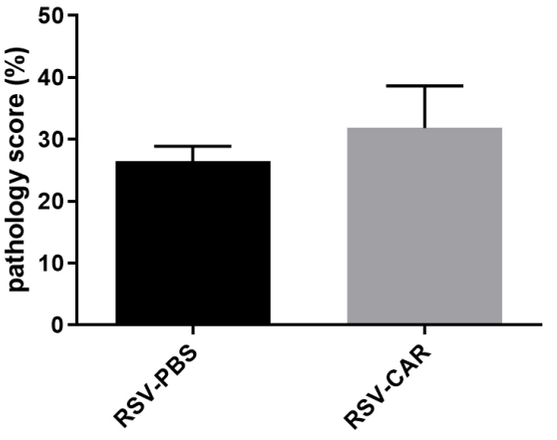

Fig. 6. The effects of carvacrol on pathology scores: lung pathology scores were insignificantly higher in the RSV-CAR group mice than the RSV-PBS group members

rol (80 mg/kg) in this study was chosen according to the literature (13), investigating another dose could be helpful.

One of the critical findings in the results of this study is the increase in the virus titer following carvacrol administration. Therefore, the finding of its possible reasons might be helpful in future studies. An assumption could be that the alternation of the cells' situations resulting from carvacrol administration made a better environment for viruses to infect cells and replicate in them however; its mechanism is unknown. Another reason could be that because of the immunmodulatory effect of carvacrol, it can reduce immune responses against RSV and permitted viruses to replicate better and infect more susceptible cells in the early days of administration. Therefore, the reduction of the inflammatory cytokines/chemokines might delay effective responses against RSV in the early stage of infection. And because of the increase in viral load, the production of inflammatory cytokines/chemokines continues in the rest days of infection that would worsen the condition. Thus, the most appropriate time to apply carvacrol might be several days after RSV infection.

The effects of Immunomodulatory agents are like a double-edge sword in viral infections. Appropriate timing and duration play a critical role (20). In some cases applying an immunomodulatory agent like corticosteroid did not have any positive effects on the outcome of different respiratory infections (21).

Although, notably, the dose of carvacrol and the time of its applying are crucial, its effects could be various in the different study contexts. For example, while Mahmoodi et al. showed that carvacrol at the concentration of 5 and 10 (mg/kg) led to a decrease of IFN-γ and increase of TGF-β levels in the experimental autoimmune encephalomyelitis mice (22), Kianmehr et al. implicated the opposite effects, in which the level of IFN-γ increased, however, TGF-β decreased in mice that were sensitized by ovalbumin and treated with 75, 150, and 300 µg/mL concentrations of carvacrol (14). Silva Lima et al. indicated that carvacrol has no effects on the TNF-α level in the treated mice at a concentration of 50 and 100 mg/kg (12), but the study on the mice that faced the carrageenan-induced hyper nociceptions, showed that carvacrol caused the decrease of TNF-α level (23).

CONCLUSION

In conclusion, carvacrol at the concentration of 80 (mg/kg) did not show immunomodulatory activity to alleviate the RSV infection outcome. Further research is needed to uncover the effects of the carvacrol intervention on virus replication and immune responses following RSV infection. Many herbal remedies in use contain carvacrol. However, other active substances are included too (24), but the use of herbal remedies to treat viral respiratory infections such as RSV and COVID-19 have to be performed with caution (25).

Also, due to the SARS-COV2 pandemic and the consideration of herbal remedies (25, 26), the results of this study can be a warning in this regard so that the misuse or overuse of such herbal remedies may increase inflammation and disease outcomes in people contrary to the expected effects. Therefore, caution should be taken to use such herbal medicines so that the amount and timing of their use and the target tissue are of particular importance.

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REFERENCES

1. Schmidt ME, Varga SM. Modulation of the host immune response by respiratory syncytial virus proteins. *J Microbiol* 2017;55:161-171.
2. van Drunen Littel-van den Hurk S , Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. *Rev Med Virol* 2007;17:5-34.
3. Straliotto SM, Siqueira MM, Machado V, Maia TM. Respiratory viruses in the pediatric intensive care unit: prevalence and clinical aspects. *Mem Inst Oswaldo Cruz* 2004;99:883-887.
4. Scheltema NM, Gentile A, Lucion F, Nokes DJ, Munywoki PK, Madhi SA, et al. Global respiratory syncytial virus-associated mortality in young children (RSV GOLD): a retrospective case series. *Lancet Glob Health* 2017;5(10):e984-e991.
5. Piedimonte G, Simoes EA. Respiratory syncytial virus and subsequent asthma: one step closer to unravelling the Gordian knot? *Eur Respir J* 2002;20:515-517.
6. Collins PL, Melero JA. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. *Virus Res* 2011;162:80-99.
7. Rosenberg HF, Domachowske JB. Inflammatory responses to respiratory syncytial virus (RSV) infection and the development of immunomodulatory pharmacotherapeutics. *Curr Med Chem* 2012;19:1424-1431.
8. Tahamtan A, Askari FS, Bont L, Salimi V. Disease severity in respiratory syncytial virus infection: role of host genetic variation. *Rev Med Virol* 2019;29(2):e2026.
9. Tahamtan A, Samieipoor Y, Nayeri FS, Rahbarimanesh AA, Izadi A, Rashidi-Nezhad A, et al. Effects of cannabinoid receptor type 2 in respiratory syncytial virus infection in human subjects and mice. *Virulence* 2018;9:217-230.
10. Hayatdavoudi P, Khajavi Rad A, Rajaei Z, Hadjzadeh MA. Renal injury, nephrolithiasis and Nigella sativa: a mini review. *Avicenna J Phytomed* 2016;6:1-8.
11. Friedman M. Chemistry and multibeneficial bioactivities of carvacrol (4-isopropyl-2-methylphenol), a component of essential oils produced by aromatic plants and spices. *J Agric Food Chem* 2014;62:7652-7670.
12. Lima Mda S, Quintans-Júnior LJ, de Santana WA, Kaneto CM, Pereira Soares MB , Villarreal CF. Anti-inflammatory effects of carvacrol: evidence for a key role of interleukin-10. *Eur J Pharmacol* 2013;699:112-117.
13. Feng X, Jia A. Protective effect of carvacrol on acute lung injury induced by lipopolysaccharide in mice. *Inflammation* 2014;37:1091-1101.
14. Kianmehr M, Rezaee A, Mahmoudi M, Ghorani V, Boskabady MH. T helper cells subtypes and their cytokine gene expression affected by carvacrol in sensitized mice administered during sensitization period. *J Cell Biochem* 2019;120:5343-5354.
15. Salimi V, Hennus MP, Mokhtari-Azad T, Shokri F, Janssen R, Hodemaekers HM, et al. Opioid receptors control viral replication in the airways. *Crit Care Med* 2013;41:205-214.
16. Shirey KA, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC, et al. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4Ralpha-, TLR4-, and IFN-beta -dependent. *Mucosal Immunol* 2010;3:291-300.
17. de Carvalho FO, Silva ÉR, Gomes IA, Santana HSR, do Nascimento Santos DKD, de Oliveira Souza GP, et al. Anti-inflammatory and antioxidant activity of carvacrol in the respiratory system: a systematic review and meta-analysis. *Phytother Res* 2020;34:2214-2229.
18. Rajabli N, Williamson L, Nimmer PS, Kelly-Worden M, Bange JS, Ho Y, et al. The dangers of sublethal carvacrol exposure: increases in virulence of *Bacillus cereus* during endophthalmitis. *Int J Biochem Mol Biol* 2018;9:11-21.
19. Stojanović NM, Stevanović M, Randjelović P, Mitić K, Petrović V, Sokolović D, et al. Low dose of carvacrol prevents rat pancreas tissue damage after L-arginine application, while higher doses cause pancreatic tissue impairment. *Food Chem Toxicol* 2019;128:280-285.
20. Alunno A, Carubbi F, Rodríguez-Carrio J. Storm, typhoon, cyclone or hurricane in patients with COVID-19? beware of the same storm that has a different origin. *RMD Open* 2020;6(1):e001295.
21. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. *Lancet* 2020;395:473-475.
22. Mahmoodi M, Amiri H, Ayoobi F, Rahmani M, Taghipour Z, Ghavamabadi RT, et al. Carvacrol ameliorates experimental autoimmune encephalomyelitis through modulating pro-and anti-inflammatory cytokines. *Life Sci* 2019;219:257-263.
23. Guimarães AG, Xavier MA, de Santana MT, Camargo EA, Santos CA, Brito FA, et al. Carvacrol attenuates mechanical hypernociception and inflammatory response. *Naunyn Schmiedebergs Arch Pharmacol* 2012;385:253-263.
24. Khazdair MR, Anaeigoudari A, Hashemzehi M, Mohebbati R. Neuroprotective potency of some spice herbs, a literature review. *J Tradit Complement Med* 2018;9:98-105.
25. Yang Y. Use of herbal drugs to treat COVID-19 should be with caution. *Lancet* 2020;395:1689-1690.
26. Wang Y, Zeng X, Zhao Y, Chen W, Chen YZ. The pros and cons of traditional Chinese medicines in the treatment of COVID-19. *Pharmacol Res* 2020;157:104873.