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Antimicrobial Activities of the Combined Use of *Cuminum Cyminum* L. Essential Oil, Nisin and Storage Temperature Against *Salmonella typhimurium* and *Staphylococcus aureus In Vitro*

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Background: Foodborne diseases are considered as major health problems in different countries. Concerns over the safety of some chemical preservatives and negative consumer reactions to them have prompted interest in natural alternatives for the maintenance or extension of food shelf life. In this respect, the combination of a plant essential oil and nisin has used for controlling the growth of foodborne pathogens as natural food preservative using the mathematical model.
Objectives: The purpose of this study was to determine the effect of different concentrations of *Cuminum cyminum* L. essential oil (0, 15, 30 and 45 μL/100 mL) and nisin (0, 0.5 and 1.5 μg/mL) combination at different temperatures (10, 25 and 35°C) on growth of *Salmonella typhimurium* and *Staphylococcus aureus* in the Brain-Heart Infusion (BHI) broth model. The concentrations of 0 μL/100 mL for essential oil and 0 μg/mL for nisin imply the negative control.
Materials and Methods: A multivariate variance experiment was performed. To assess the effect of essential oil, nisin and the incubation temperature on growth probability (log P%) of *S. typhimurium* and *S. aureus*, four concentrations of *C. cyminum* L. essential oil (0, 15, 30 and 45 μL/100 mL), three concentrations of nisin (0, 0.5 and 1.5 μg/mL) and three storage temperatures (10, 25 and 35°C) were considered.
Results: The growth of *S. typhimurium* was significantly decreased by the concentration of essential oil ≥ 30 μL/100 mL in combination with nisin ≥ 0.5 μg/mL at temperature = 10°C ($P < 0.05$). Also, in combination of the essential oil ≥ 15 μL/100 mL and nisin ≥ 0.5 μg/mL at temperature ≤ 25°C, the growth of *S. aureus* was significantly reduced ($P < 0.05$).
Conclusions: These results indicate that the combination of essential oil and nisin inhibits the growth of *S. typhimurium* and *S. aureus* bacteria and there is the possibility of using them as substitutes for chemical food preservatives. Moreover, the model (log P%) in this study can be a good tool for the reduction of microbiological hazards in food industry.

Keywords: Antimicrobial Activity, Essential Oil; Nisin; *Salmonella typhimurium*; *Staphylococcus aureus*

1. Background

Foodborne diseases are considered as major health problems in different countries (1). Scallan et al. estimated that major known pathogens and unspecified agents transmitted by food result in an estimated 47.8 million illnesses, 127,839 hospitalizations, and 3037 death each year in the United States (2). *Salmonella typhimurium* and *Staphylococcus aureus* are known to have caused outbreaks through consumption of foods (3, 4). Concerns over the safety of some chemical preservatives and negative consumer reactions to them have prompted interest in natural alternatives for the maintenance or extension of food shelf life. Particular interest has focused on the potential applications of plant essential oils (5-7). In this respect, the plant essential oils and their components could use for controlling the growth of foodborne pathogens and food spoilage microorganisms as natural food preservatives (8, 9).

Cuminum cyminum L. (Cumin) from *Apiaceae* family is native to the Mediterranean region and has long been used in traditional medicine (10, 11) and many species of traditional medicinal plants of Iran are classified in this family, such as Coriander (*Coriandrum sativum*), Bilhar (*Doremaaucheri*), and Schlecht (*Ferulago angulata* boiss.). Cumin seeds have distinctive flavor and strong, warm aroma due to its essential oil content (12, 13). Nowadays, it is cultivated in China, India, Indonesia, Iran, North Africa, Pakistan, Sri Lanka, Syria, and Turkey. Iran has been the principal supplier of cumin in the world (14). *C. cyminum* L. is mainly used where highly spiced foods are preferred. It is stomachic, diuretic, carminative, stimulant, astringent, emmenagogic and antispasmodic (10, 11, 15). The effect of *C. cyminum* L. essential oil has been well-known on growth inhibition of some fungi that have role in food

spoilage and putrefaction (16, 17). The antimicrobial effect of *C. cyminum* L. essential oil on microorganisms such as *Klebsiella pneumoniae*, *Vibrio spp*., *S. aureus*, *Bacillus cereus*, *Escherichia coli* O157:H7, *S. enteritidis*, and *Listeria monocytogenes* has been investigated in several studies (12, 13, 18, 19).

The essential oils, such as *C. cyminum* can be used as food preservatives. However, to establish the usefulness of natural antimicrobial preservatives, they must be evaluated alone and in combination with other preservative factors such as nisin; therefore, it can be determined whether there are synergistic effects (20, 21). Nisin is a ribosomal peptide (bacteriocin) that is produced by *Lactococcus-lactis* subsp. *Lactis* that was adopted by joint FAO/WHO expert committee as an effective natural food preservative in food industry, such as dairy and meat products. Maximum limit of nisin is 12.5 mg/kg in food. Bactericidal activities of nisin occur by two mechanisms, create pore in bacterial cell wall and inhibition of peptidoglycan biosynthesis (20, 22-25).

In recent years the mathematical *in vitro* models to predict the growth kinetic, e.g. growth/ no growth and time-to-detection, of bacteria in defined system have been developed, so for this purpose, researches have done first in laboratory media and then in food model (26-28).

2. Objectives

The purpose of this study was to evaluate the effect of *C. cyminum* L. essential oil alone and in combination with nisin at different temperatures on growth of *S. typhimurium* and *S. aureus* in Brain-Heart Infusion (BHI) broth for 43 days. This model was designed according to previous studies (28-30).

3. Materials and Methods

3.1. Plant Material

C. cyminum L. (Linnean society of London Herbarium No. LIMN-HS511.1) was collected from Kerman province of Iran at flowering stage of plant in June 2013 and identified by Institute of Medicinal Plants of Tehran University, Tehran, Iran.

3.2. Extraction of Essential Oil

Air-dried seeds and aerial parts of the plant were subjected to steam distillation for 2 hours using a Clevenger-type apparatus (Corning, Mexico). The essential oil was weighed, stored at 4°C in sealed ampoules and used within days (11-13, 15, 18).

3.3. Identification of Essential Oil Components

The C. cyminum L. essential oil was analyzed by Gas Chromatography (GC) (Thermo Quest 2000, UK) and also analyzed by Gas Chromatography-Mass Spectrometery (GC–MS) (Thermo Quest/ Finnigan, UK) equipped with silica capillary column (30 mm × 0.25 mm inner diameter; film thickness of 0.25 μm) and coated with DB-5. The MS was scanned in the electron impact mode, using ionization energy of 70e-V. The Retention Index (RI) for each of the separated materials was calculated based on the extraction time and identification of the major components of essential oil was confirmed by comparing their relative KovatsRI and mass spectra with the standard of the National Institute of Standards and Technology (NIST 05) mass spectral library and those have reported in the literature (15, 18, 29).

3.4. Nisin Preparation

Nisin containing 2.5% active nisin was purchased from (Sigma Aldrich, UK). Nisin stock solution was prepared with 0.02 mol/L 1 HCl (pH 1.6), and was filter-sterilized through a 0.22 μm disposable sterile syringe filter (Merck, Germany).

3.5. Test Organism

Lyophilized cultures of *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 were obtained from department of microbiology, faculty of veterinary medicine, University of Tehran, Iran. Consequently, the lyophilized culture was grown in a tube containing 10 mL of BHI broth (Merck, Germany) twice and incubated at 35°C for 18 hours. Then cultures were stored at 20°C with 10% sterile glycerol in 1 mL microtubes and their subcultures were used as working cultures.

3.6. Inoculums Preparation

Salmonella typhimurium and *S. aureus* inoculums were prepared by transferring from working culture to the sterile BHI broth. They were grown for 18 hours at 35°C, and then second subculture was prepared by incubation for 18 hours at 35°C. Different values from second cultures were transferred to the 13 × 100 mm cuvettes containing 5 mL sterile BHI broth and they were adjusted to Optical Density (OD) (absorbance) of 0.02 and 0.01 at 600 nm for *S. typhimurium* and *S. aureus* cultures respectively, using a spectronic 20 spectrophotometer (Boeckel and Co., Germany). Thus, bacterial suspensions were adjusted with the same concentration to contain approximately 1×10^7 CFU/mL. The bacterial suspensions were enumerated by performing 10-fold serial dilutions and surface plating culture on BHI agar. Then colony counting was done after a 24-hour incubation period at 35°C (28).

3.7. Preparation of Broth Substrate

The BHI broth with 5% (v/v) di-methylsulfoxid (DMSO, Merck Schuchardt OHG, Hohenbrunn, Germany) and 0.05% (w/v) agar (Merck, Germany) was prepared as a stabilizer in three 4000 mL screw-capped glass bottles (Sigma-Aldrich Co., USA) and then autoclaved at 121°C for 15 minutes (31).

3.8. Preparation of In Vitro Model

A multivariate variance experiment was performed. To assess the effect of essential oil, nisin and incubation temperature on growth probability (log P%) of *S. typhimurium* and *S. aureus*, four concentrations of *C. cyminum* L. essential oil (0, 15, 30 and 45 μL/100 mL), three concentrations of nisin (0, 0.5 and 1.5 μg/mL) and three storage temperatures (10, 25 and 35°C) were considered. Thus, 36 different levels (4 × 3 × 3) were used for each microorganism. For each combination of essential oil-nisin-temperatures, 8 bacterial dilution of 10^5 to 10^{-2} CFU/mL from *S. typhimurium* and *S. aureus* in the 16 × 100 mm screw-capped tubes contained 9 mL BHI broth were obtained and stored for up to 43 days. Time to visible turbidity at 18 intervals (1, 2, 3, 4, 5, 6, 7, 8, 13, 16, 19, 22, 25, 28, 31, 34, 37 and 43 days) was recorded. All of the experimental processes were done for triplet repeat.

3.9. Calculation of Probability of Growth (Log P%)

The Log P% was calculated from total tubes (out of 36) for each essential oil-nisin-temperature combination showing visible growth up to a certain observation time, using 3 × 8 Most Probable Number (MPN) method (27). The fraction of the inoculum which was inhibited by each broth environment up to that time was estimated from the formula $\log_{10}$I/G, where I was the number of cells inoculated in the highest cell concentration tube and G was the MPN of cells in the same tube, which managed to grow. The probability percentage (P%) of any given cell initiating growth under each broth environment within a certain period of time was defined as P% = 100/antilog ($\log_{10}$I/G). The number of cells needed to initiate visible growth for each essential oil-nisin-temperature combination was estimated from cells needed = 100/P% (28).

3.10. Statistical Analysis

The main and interactive effects of independent variables of temperature, concentration of nisin, essential oil and storage time (day) on dependent variables of log P% of S. typhimurium and *S. aureus* were evaluated by analysis of variance (ANOVA) using IBM SPSS Statistics software version 22 (27).

4. Results

4.1. Chemical Compositions of Cuminum cyminum L. Essential Oil

In this study, the yield of the essential oil of air-dried seeds and aerial parts of plant samples of *C. cyminum* L. was 3.56% (v/w). The results for the percentages of the components of essential oil (as determined by GC/MS) are shown in Table 1. From the *C. cyminum* L. essential oil, 16 components were identified, representing 91.96% (area percent) of the total essential oil, which δ-Terpinene (22.30%) and Cuminaldehyde (21.09%) were the major components. Other important components were < ortho > Cymene (12.71%), β-Pinene (8.46%) and < para > Cymene-7-ol (6.67%) (Table 1).

Table 1. Main Components and the Relative Contents of *Cuminum cyminum* L. Essential Oil

Retention Index	Components				Percent
	Monoterpenes Hydrocarbons	Oxygenated Monoterpenes	Sesquiterpene Hydrocarbons	Others	
11.27	α-Pinene				1.26
13.55	β-Pinene				8.46
14.21	Myrcene				0.88
14.83	Phellandrene				1.59
16.11	< ortho > Cymene				12.71
16.29	Limonene				4.63
16.66	β-Ocimene < Z >				1.17
18.09	δ-Terpinene				22.30
19.18	α-Terpinolene				0.94
27.03		Cumin aldehyde			21.09
28.83		Menth-1-en-7-al < para >			5.99
29.16		< para > Cymen-7-ol			6.67
34.60			Caryophyllene		0.87
36.91			β-Acoradien		1.27
38.74				Myristicin	1.20
42.72				Dill apiole	0.93
Total identified	53.94	33.75	2.14	2.13	91.96

4.2. Factor Effects

In the present study, the log P% was used in a factorial design study to quantify the effects of essential oil-nisin-temperature and day (for log P%) on the bacterial growth responses in BHI broth (28, 29). Table 2 represents the values of log P% at day to reach the maximum probability of growth for *S. typhimurium* and *S. aureus* and cells needed as affected by combinations of essential oil, nisin, and temperature in BHI broth model.

The essential oil without nisin (nisin = 0.0 μg/mL), at all temperature (Temperature ≤ 35°C) could not obviously affect the growth of the *S. typhimurium* in this study. Whereas, essential oil ≥45 μL/100 mL without nisin, at all temperature (Temperature ≤ 35°C) have a significant inhibitory action on *S. aureus* in this study (log P% = -3.52 to 2.97 and cells needed = 0.11 to 3.33×10^5).

The nisin ≥ 1.5 μg/mL without essential oil (essential oil = 0 μL/100 mL) and temperature ≤ 25°C showed an inhibitory effect on *S. typhimurium*. However, the inhibitory effect of nisin on *S. aureus* was stronger and could affect growth of it at nisin ≥ 1.5 μg/mL without essential oil (essential oil = 0 μL/100 mL) at all temperature (temperature ≤ 35°C). Moreover, the inhibitory effects of essential oil and nisin were enhanced by decreasing the storage temperature to 10°C ($P \leq 0.05$).

The combination of essential oil and nisin strongly affected the growth of *S. typhimurium* and *S. aureus* at the different temperatures. By applying essential oil ≥ 30 μL/100 mL and nisin ≥ 0.5 μg/mL at temperature = 10°C, a significant decrease of logP% was observed for *S. typhimurium* (logP% = 1.38 to 3.66 and cells needed = 0.02 to 4.17) ($P \leq 0.05$). Also, the growth of *S. aureus* in the same conditions was completely inhibited (logP% = -3.52 and cells needed = 3.33×10^5).

At temperature ≥ 25°C, the combination of essential oil and nisin (essential oil ≥ 15 μL/100 mL, nisin ≥ 0.5 μg/mL and temperature ≤ 25°C) could reduce the growth of *S. aureus* (logP% = 1.38 to 1.63 and cells needed = 2.33 to 4.17).

According to the results of ANOVA (P values), the logP% of *S. typhimurium* and *S. aureus* was affected significantly ($P < 0.01$) by essential oil, nisin, temperature, day, two-way interactions of essential oil × temperature, nisin × temperature, and essential oil × nisin and three-way interactions of essential oil × nisin × temperature.

5. Discussion

Articles of medicinal plant have been reported a better antibacterial effect of essential oil in comparison with alcoholic extract, hydro-extract and powder of plant. This antibacterial effect of essential oils is related to main components of them (8). These components, with a chemical structure including an aromatic ring, are able to disintegrate the outer membrane of bacteria and increase the permeability of the cytoplasmic membrane to the ATP (32). The main component of *C. cyminum* L. essential oil is cuminaldehyde or 4-isopropylbenzaldehyde and other principle active components are alpha and beta pinene, alcohol of cumin, di-pentene, para-cymene, and beta-phellandrene (18, 33, 34). These components, which have the foresaid structure are chiefly responsible for the antibacterial properties of essential oils (8). The profile of the *C. cyminum* L. essential oil was also in agreement with values reported from other studies. Nanasombat and Lohasupthawee concluded that the major components of this essential oil arecuminaldehyde (20.72%) and monoterpene hydrocarbons (e.g. β-pinene, γ-terpinene, p-cymene) (34). Derakhshan reported components of essential oil including cuminaldehyde (25.2%), ρ-mentha-1,3-dien-7-al (13%), ρ-mentha-1,4-dien-7-al (16.6%), δ-terpinene (19%), ρ-cymene (7.2%) and β-pinene (10.4%).Other components were α-thujene (0.2%), α-pinene (0.6%), sabinene (0.7%), myrcene (0.8%), α-phellandrene (0.4%), β-phellandrene (0.7%) and ρ-menth-3-en-7-al (5.1%) (18). Similar findings have been reported by Gachkar et al. as a result of GC-MS analyses, *C. cyminum* contained α-pinene (29.2%), 1, 8-cineole (17.9%), and linalool (10.4%) (12).

The different qualitative and quantitative chemical-compositions of these essential oils with respect to previous investigations could be related firstly and foremost to the different environmental conditions, genetics (degree of hybridization), geographical origin and a harvest period (35, 36).

Plant essential oils are potentially useful sources of antimicrobial components (5-9). However, findings reported from studies of essential oils antimicrobial effects are difficult to compare, because the test methods, bacterial strains and source of antimicrobial agents are different (29, 37, 38).

In many researches, the antimicrobial activity of *C. cyminum* L. on different bacteria such as *P. aeroginosa*, *Vibrio spp*., *K. pnomonia* and others have surveyed and concluded that *C. cyminum* L. along with the effect of broad-spectrum antibiotics inhibit the growth of pathogenic bacteria (13, 18, 39).

Gachkar et al. surveyed kinetics of death of *E. coli*, *S. aureus* and *L. monocytogenes* exposed to the Minimum Bactericidal Concentration levels of *C. cyminum* L. and *Rosmarinus officinalis* essential oils that *C. cyminum* L. essential oil exhibited stronger antimicrobial activity than did *R. officinalis* essential oil. They suggested that *C. cyminum* L. essential oil may be considered as potent agents in food preservation (12).

Chaudhry and Tariq investigated the antibacterial activity of different essential oils such as aqueous *Nigella sativa* L., *C. cyminum* L., and *Papaver somniferum* L. against 188 bacterial isolates and the highest antibacterial potential was observed for *C. cyminum* L. (33). Derakhshan et al. also found that growth of *K. pneumonia* strains exposed to sub-MICs of *C.cyminum* L. extracts resulted in cell elongation and repression of capsule expression (18). Hajlaoui et al. reported the high effectiveness of *C. cyminum* L. essential oil against *Vibrio spp*. Strains and their antibacterial, antifungal and antioxidant components can be used for therapeutic or nutraceutical industries (13).

Table 2. Log_{10} Probability Percentage of Growth Initiation at the Day to Reach the Maximum Probability of Growth for *S. typhimurium* and *S. aureus*, the Number of cells needed to Initiate Growth During 43 Days of Storage in BHI Broth, as Affected by Combinations of *Cuminum cyminum* L. Essential oil, Nisin, and Temperature

Temperature, °C	Essential oil, µL/100 mL	Nisin, µg/mL	*S. typhimurium*			*S. aureus*		
			Day	Growth Initiation, %	Cells Need	Day	Growth Initiation, %	Cells Need
10	0	0.0	12	3.66	0.02	16	-1.34	2.17 × 103
10	0	0.5	25	3.66	0.02	16	-1.34	2.17 × 103
10	0	1.5	25	2.97	0.11	> 43	-3.44	2.78 × 105
10	15	0.0	25	3.66	0.02	16	-1.34	2.17 × 103
10	15	0.5	31	3.66	0.02	16	-2.64	4.35 × 104
10	15	1.5	31	2.97	0.11	> 43	-3.52	3.33 × 105
10	30	0.0	25	3.66	0.02	16	-1.34	2.17 × 103
10	30	0.5	31	3.38	0.04	> 43	-3.52	3.33 × 105
10	30	1.5	31	1.38	4.17	> 43	-3.52	3.33 × 105
10	45	0.0	28	3.66	0.02	> 43	-3.52	3.33 × 105
10	45	0.5	31	2.97	0.11	> 43	-3.52	3.33 × 105
10	45	1.5	37	1.38	4.17	> 43	-3.52	3.33 × 105
25	0	0.0	1	3.66	0.02	2	3.38	0.04
25	0	0.5	1	3.66	0.02	2	3.38	0.04
25	0	1.5	3	3.18	0.07	3	1.63	2.33
25	15	0.0	1	3.66	0.02	2	3.38	0.04
25	15	0.5	2	3.66	0.02	3	1.63	2.33
25	15	1.5	3	2.88	0.13	4	1.38	4.17
25	30	0.0	1	3.66	0.02	2	3.38	0.11
25	30	0.5	2	3.66	0.02	3	1.38	4.17
25	30	1.5	3	2.30	0.50	3	1.38	4.17
25	45	0.0	1	3.66	0.02	3	2.97	0.11
25	45	0.5	2	3.66	0.02	4	1.38	4.17
25	45	1.5	4	2.18	0.67	4	1.38	4.17
35	0	0.0	1	3.66	0.02	2	4.04	0.01
35	0	0.5	1	3.66	0.02	2	4.04	0.01
35	0	1.5	2	3.66	0.02	3	1.63	2.33
35	15	0.0	1	3.66	0.02	2	4.04	0.01
35	15	0.5	2	3.66	0.02	2	1.63	2.33
35	15	1.5	2	2.97	0.11	3	1.38	4.17
35	30	0.0	1	3.66	0.02	2	4.04	0.01
35	30	0.5	2	3.66	0.02	3	1.38	4.17
35	30	1.5	2	2.97	0.11	3	1.38	4.17
35	45	0.0	1	3.66	0.02	3	2.97	0.11
35	45	0.5	2	3.66	0.02	3	1.38	4.17
35	45	1.5	2	2.38	0.42	3	1.38	4.17

Leistner and Goris already suggested that food preservation by multiple preservatives in small amounts was superior to preservation by a large amount of a single preservative in order to both secure microbial stability and safety (40).

Natural products and derived components from plants

may have applications in controlling pathogens in food. However, to establish the usefulness of natural antimicrobials, they must be evaluated alone or in combination with other hurdles. Moreover, Valero and Salmeron determined the synergistic effects in order to both secure microbial stability and safety and maintain the sensory, nutritive and economic properties of the foods (38).

Our results (Table 2) showed a significant inhibitory effect of essential oil in combination with nisin on *S. typhimurium* and *S. aureus* growth in the BHI broth which is similar to the findings of Valero and Giner that showed the synergistic effect of essential oils with nisin in food model system on bacterial growth (41).

The effect of combining nisin and essential oils has studied. Nevertheless, Rajkovic et al. observed the accomplished growth inhibition of *B. cereus* and *Bacillus circulans* strains affected by antimicrobial potential of combination of supplemented nisin and carvacrol essential oil in the BHI broth model and vacuum-packed potato puree (42). Moosavy et al. reported that the combination of *Zataria multiflora* Boiss. essential oil and nisin at low concentrations exhibited a higher activity against *S. typhimurium*and *S. aureus* than individual essential oils applied at higher concentrations (43). Pajohi et al. investigated effects of the *C. cyminum* L. essential oil alone and in combination with nisin on survival of vegetative forms of *B. cereus* and *B. Subtilis* in a food model (commercial barley soup) that a synergistic effect of the essential oil in combination with the lowest concentration of nisin at 8°C was observed on the bacterial growth (44).

Misaghi and Akhondzadeh Basti showed that the growth of the *B. cereus* ATCC 11778 was completely inhibited at combinations *Z. multiflora* Boiss. essential oil $\geq$ 0.015%, nisin $\geq$ 1.5 μg/mL, temperature $\leq$ 30°C and pH $\leq$ 7.4 during 43 days of storage (30). They recommended to apply essential oils as a part of a hurdle system and to use them as antimicrobial components with other preservation techniques e.g. in combination with reduced temperature or other natural preservatives such as nisin.

Since essential oil containing mainly phenolic compounds and nisin acts on bacterial cytoplasmic membrane, their antibacterial activity could be enhanced by treatments involving combination of them (45). Therefore, synergism between essential oils and other parameters in antimicrobial action must be considered and further research is needed to evaluate the effectiveness of combined essential oils in the current and other food systems as well as by using active packaging, in order to assess their performance as natural antimicrobial agents in food preservation and safety (46).

Our results in this study indicate the good potential antimicrobial effect of *C. cyminum* L. essential oil and nisin combination on *S. typhymurium* growth at 10°C and *S. aureus* growth at 10°C and 25°C in the BHI broth during 43 days. Such models offer a cost-effective approach to control the microbial growth response in foods.

Authors' Contributions

Study concept and design: Hamid Reza Tavakoli, Zohreh Mashak, and Bizhan Moradi. Analysis and interpretation of data: Hamid Reza Tavakoli, Zohreh Mashak, and Bizhan Moradi. Drafting of the manuscript: Bizhan Moradi and Hamid Reza Sodagari. Critical revision of the manuscript for important intellectual content: Zohreh Mashak, Hamid Reza Tavakoli, Bizhan Moradi and Hamid Reza Sodagari. Statistical analysis: Bizhan Moradi.

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