Original Article

The Chemical Composition and Antibacterial Effect of Essential Oils of Rosemary and Basil in Milk

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ABSTRACT

Background: According to the diverse side effects of antibiotics, new and natural antibacterial substances are needed to treat bacterial diseases, and one of these substances is the essential oils (EOs) of medicinal plants. Milk fat and protein may reduce the antimicrobial impact of EOs.

Objectives: This study aims to investigate the antibacterial activity of rosemary and basil EOs compared to lincospectinomycin antibiotic against three mastitis-causing bacteria, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli* in milk media.

Methods: Chemical compounds in EOs were identified by gas chromatography. The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of EOs were studied using the tube dilution method, and the growth curve of bacteria was studied at 0, 6, 10, and 24 h.

Results: The most crucial rosemary compounds were carene (45.11%) and eucalyptus (20.62%), and those of basil were estragol (70.42%) and carene (17.99%). The MIC and MBC of rosemary were lower than those of lincospectinomycin, and those of basil were similar to those of lincospectinomycin. At 6-h, the bacterial reduction of *E. coli* and *S. agalactiae* was significant, and the population reduction of rosemary was significant for *S. aureus*. At 24 h, rosemary and basil significantly diminished the bacterial count of *S. aureus*, and basil significantly decreased the *S. agalactiae count*.

Conclusion: The antibacterial effects of EOs are acceptable, and clinical studies are recommended to treat other diseases, including mastitis.

Keywords: Antibacterial effect, Basil, Mastitis, Milk, Rosemary

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Introduction

Bovine mastitis affects dairy cows and is a major economic threat to the dairy industry worldwide. This is also a potential public health concern. The most common causes of this disease are *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus agalactiae* (Zhu et al., 2016). For many years, administering antibacterial agents directly into the udder has been the primary approach for treating and preventing mastitis in dairy animals. The emergence of antibiotic-resistant bacteria due to their use in animals is a major concern with significant public health implications (Alekish et al., 2017; Foroutan et al., 2021; Abbasi et al., 2024). There is global concern regarding the widespread use of bacteria resistant to multiple drugs and the low effectiveness of newly developed antibiotics. Therefore, using natural resources, particularly essential oils (EOs), has been strongly emphasized to discover new antibacterial agents (Sharifi-Rad et al., 2020; Gaddafi et al., 2023).

Ocimum basilicum (basil), especially its aromatic leaves, has good medicinal effects and has been used in traditional medicine as a vermifuge, tonic, antispasmodic, diuretic, and for the treatment of infections of the upper respiratory tract (Al Abbasy et al., 2015). Several researchers have explored the potent antimicrobial properties of basil EOs. Basil EO has shown potent antibacterial effects against gram-negative and gram-positive bacteria (Rezzoug et al., 2019; De Martino et al., 2021; da Silva et al., 2022).

The aerial parts of Rosmarinus officinalis, also known as rosemary, contain EOs and phenolic compounds with various pharmacological effects, including antibacterial, anti-inflammatory, and antiviral properties. The key constituents of EO are 1,8-cineole, camphor, borneol, and β-caryophyllene. The essential oil composition can vary depending on the season, climate, land, soil, and developmental stage (Oliveira et al., 2019; Ali Hasan and Al-Rikaby, 2022; Rathore et al., 2022).

The fat, starch, and albumin in milk may interact with antibacterial compounds and decrease the bioavailability of EOs (Burt, 2004). Hence, it is crucial to evaluate the effectiveness of EOs in killing bacteria in milk before using them as an intramammary infusion for mastitis treatment. Assessing the antibacterial properties of EOs in milk is more challenging than in laboratory media. Although rosemary and basil are rich in EOs and numerous studies have been conducted about their antibacterial effects in laboratory mediums, research on their antibacterial effects in milk is scarce. Therefore, this study aims to investigate the antibacterial activity of EOs in milk, which simulates the udder environment.

Materials and Methods

Rosemary and basil EOs were purchased from Dorrin Golab Company, Kashan City, Iran.

Chemical composition identification of the EOs

Analysis was conducted using an Agilent 7890B gas chromatograph. The chromatograph was coupled to a mass spectrometer (Model 5977A, Agilent Technologies, USA). A HP-5MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm ID 0.25 um, Agilent Technologies) was used to separate the compounds in the sample. The injector temperature was set at 270 ˚C. The oven temperature program started at 60 ˚C and was increased to 200 ˚C at a rate of 5 ˚C/min. Helium was used as the carrier gas, which helped to move the sample through the column. The injection volume was one microliter. The mass spectrometer scanned a range of 35-500 m/z, and the interface temperature was set to 280 ˚C.

Bacterial strain

The effectiveness of EOs in combating three major mastitis bacteria, *S. agalactiae* (American Type Culture Collection [ATCC] 13813), *E. coli* (ATCC 25922), and *S. aureus* (ATCC 9144), was tested. Lyophilized cultures containing bacteria were obtained from the Persian Type Culture Collection in Tehran Province, Iran (PTCC). Tubes containing 10 mL Tryptic Soy Broth (TSB) (Biolife, Milano, Italy) were incubated for 18-20 hours at 37 ˚C, twice for growth. The cultures were mixed with sterile glycerin at a 1:5 ratio and stored at -20 ˚C. Twice the culture in TSB at 37 ˚C for 20 h was used to obtain fresh bacteria. The cultures were stored at 4 ˚C after streaking on Tryptic Soy Agar (TSA) slants (Biolife, Milano, Italy) and incubated (Basti et al., 2007).

Inoculum preparation

Cells were transferred from the working cultures to TSB tubes and incubated at 35 ˚C for 18 h to obtain the bacterial inoculum. The subcultures were prepared and incubated for 18 h at 35 ˚C. A Biochrom Ltd. spectrophotometer (Libra S12, Cambridge, London) was utilized to adjust cultures to OD 0.1 at 600 nm. This resulted in a cell concentration of 4.1×10^7 CFU/mL for *S. agalactiae*, 1.2×10^8 CFU/mL for *S. aureus*, and 3.6×10^6 CFU/mL for *E. coli*. Counting cells in the suspensions was performed by duplicate plating and incubating from tenfold serial dilutions on TSA (Basti et al., 2007). Finally, 1:500 dilutions of the primary inoculum were used as working inocula.

Milk

Free antibiotic raw milk was autoclaved for 15 minutes at 121 °C.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Dimethylsulphoxide (DMSO) (Sigma, Germany) was used for the dilution of EOs at a ratio of 1:1. This dilution was then passed through a filter to sterilize it and used for antibacterial analysis. MIC and MBC were determined using a modified protocol for broth dilution (CLSI, 2015). The whole autoclaved milk was used as the growth medium. Two-fold serial dilutions of the oil (10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156%) were performed in milk to determine MIC. Thereafter, 100 µL of the bacterial inoculum was added, and after overtaxing, the vials were incubated for 24 h at 37 °C. To enumerate inoculated bacteria, 100 µL of each vial was plated on a TSA plate and incubated for 24 h at 37 °C. The lowest concentration without visible growth was considered the MBC, and the following concentration was defined as the MIC. To ensure that the autoclaving process was successful, a negative control (milk culture alone) was employed. Milk-containing bacteria were used as positive controls to document bacterial growth in milk. DMSO was the vehicle control to evaluate this solvent's possible antibacterial effect.

Bactericidal kinetics of the oils

The experiment involved inoculating sterile milk with different pathogens and exposing them to sub-MIC of EOs, similar to the MIC tests. Inoculated milk without EO was used as a control sample. After incubation at 37 °C for 24 h, the bacterial population was counted at 0, 6, 10, and 24 h of incubation following 0.1 mL plating of the nine serial dilutions (1:10 in normal saline). All the treatments were performed twice to ensure accuracy. Growth curves were plotted by recording bacterial count (measured in $\log_{10}$ CFU/mL) against the elapsed time (measured in hours).

Statistical analysis

All experiments were conducted in duplicate. Data were analyzed using SPSS software, version 18 (IBM Corp., Armonk, NY, USA) with analysis of variance and Tukey's test at a $P<0.05$.

Results

Chemical composition of the EOs

Gas chromatography-mass spectrometry (GC/MS) analysis revealed that the essential oil of rosemary contained 3-carene as the major constituent, with a concentration of 45.11%, followed by eucalyptol (1,8-cineol) at 20.62%, and levoverbenone at 5.91%. In basil oil, estragol (70.42%), 3-carene (17.99%), and eucalyptol (8.61%) were the main compounds (Tables 1 and 2).

MBC and MIC

The effects of rosemary and basil on *S. aureus* and *E. coli* and the effect of basil on *S. agalactiae* were similar to those of lincospectinomycin. However, rosemary's effect on *S. agalactiae* was higher than that of lincospectinomycin (Table 3).

Time kill assay

Figures 1, 2 and 3 show the impact of rosemary and basil on milk bacteria. At 6-h, the population of *S. agalactiae* and *E. coli* bacteria was significantly reduced and population reduction of rosemary was significantly for *S. aureus*. At 24 h, rosemary and basil significantly diminished the bacterial count of *S. aureus*, and basil significantly decreased the *S. agalactiae* count.

Discussion

The antimicrobial efficacy of different EOs is frequently assessed using the broth dilution method (Hood et al., 2003). However, in the present study, milk was used instead of broth to simulate the udder environment. Due to their hydrophobic nature, the presence of lipophilic molecules, including lipids in milk, may challenge the antibacterial activity of EOs against mastitis pathogens (Burt, 2004).

This study showed that carene, eucalyptol, and Levoverbenone are the major components of rosemary EO. Gachkar et al. showed a strong antibacterial effect of rosemary from Iran against *Listeria monocytogenes*, *S. aureus*, and *E. coli* (MBCs: 2-4 µg/mL), which was attributed to camphor, verbenone, and borneol (Gachkar et al., 2007). In another study from Iran, the most compounds of 7 rosemary populations were eucalyptol (5.63%-26.89%), camphor (66.1%-24.82%), and alpha-pinene (14.69%-20.81%) (Bajalan et al., 2017). A study reported a moderate antimicrobial activity of rosemary oil from Turkey (MBCs ranging from 2.5 to 20 µg/mL).

Table 1. Chemical composition (relative % of peak area) of EO of rosemary determined by GC-MS analyses

No.	Components	Retention Time (m)	Area Sum%
1	3-Carene	4.116	45.11
2	Camphene	4.415	4.34
3	Cymene	5.895	3.15
4	D-Limonene	6.003	3.94
5	Eucalyptol	6.085	20.62
6	Linalool	7.639	2.29
7	(+)-2-Bornanone	8.875	5.01
8	endo-Borneol	9.52	5.2
9	L-.alpha.-Terpineol	10.117	2.22
10	Levoverbenone	10.423	5.91
11	Thymol	12.805	2.21

This was attributed to the high 1,8-cineol content (Celiktas et al., 2007). The main components of rosemary EO from Spain and Morocco were reported to be camphor, alpha-pinene, and eucalyptol (Diass et al., 2021; Melero-Bravo et al., 2022). Alpha pinene (75.4%-18.2%) and eucalyptol (15.6%-3.5%) were the most constituents in all periods of samplings of rosemary (Serralutzu et al., 2020). Eucalyptus was reported as one of the main compounds in the present study and the above studies. However, most studies did not observe camphor and alpha-terpinene, which were reported in the present study.

In this study, the major constituents of basil EO were stragole (methyl chavicol), carene, and eucalyptol. The main components of an Iranian basil EO are methyl chavicol, linalool, and epi-α-cadinol in the purple cultivar and methyl chavicol, geranial, and neral in green cultivars (Sajjadi, 2006). In another study from Armenia, the major constituents of basil were methyl chavicol and linalool (Avetisyan et al., 2017). The composition of basil oil from Italy is affected by the season the plants are harvested. For example, the essential oil obtained from plants harvested in May was mainly composed of linalool, whereas the October sample contained eugenol as the main constituent. Various factors may cause chemical differences among different geographic regions. These factors may include solar radiation, shading, soil quality, temperature, and other factors that may influence metabolic pathways or genes responsible for producing volatiles and terpenes (da Silva et al., 2022).

Table 2. Phytochemical components (relative % of peak area) of basil EO assessed by GC-MS analyses

No.	Components	Retention Time (m)	Area Sum%
1	3-Carene	4.109	17.99
2	o-Cymene	5.895	0.97
3	D-Limonene	6.004	1.29
4	Eucalyptol	6.078	8.61
5	Linalool	7.64	0.71
6	Estragole	10.246	70.42

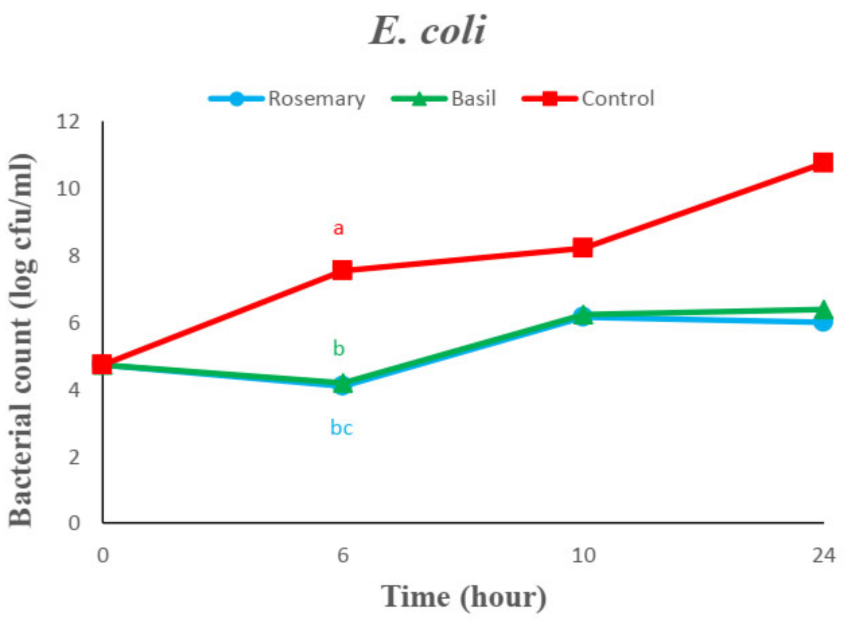

Figure 1. Growth curve of *E. coli* after exposure to 0% (control, ■) and sub-MIC of rosemary (●) and basil (▲) EO

Notes: Values marked with different letters simultaneously show significant (P<0.05) differences.

The MIC and MBC of rosemary and basil EOs against bacteria have not been reported in milk, but different values have been reported in synthetic laboratory media. In a study, MIC and MBC of 156 mg/mL were reported for rosemary essential oil against multidrug-resistant *S. aureus* (Esmael et al., 2020). In another study, the MBC and MIC of rosemary EO against multidrug-resistant *S. aureus* were 0.03% and 0.1%, respectively, and against *E. coli* , were 0.3% and 0.5%, respectively (Jiang et al., 2011). MBC and MIC of basil essential oil against *E.*

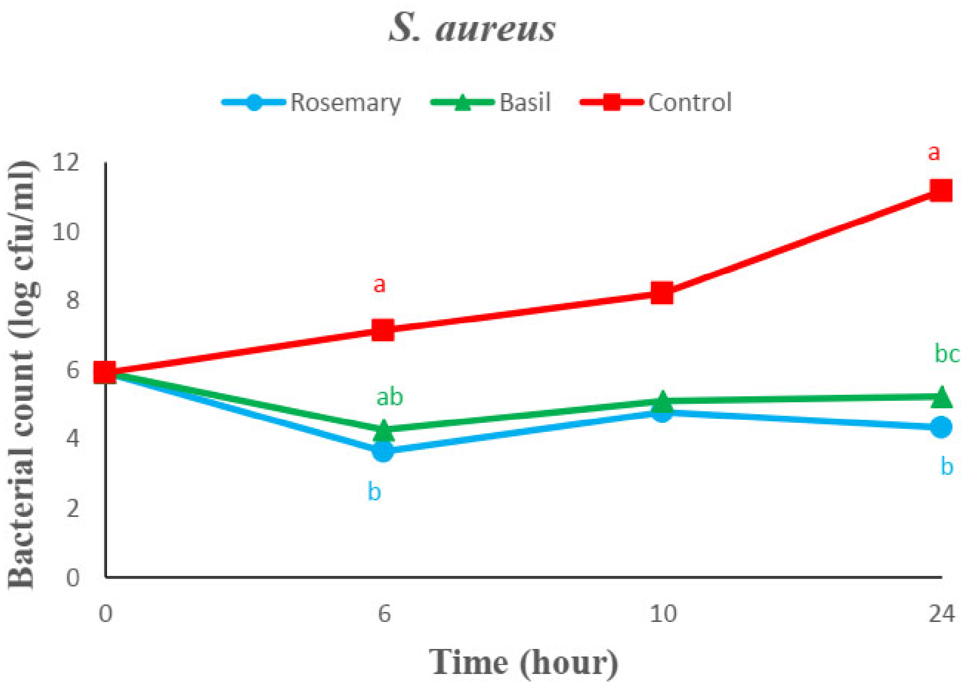

Figure 2. Growth curve of *S. aureus* after exposure to 0% (control, ■) and sub-MIC of rosemary (●) and basil (▲) EO

Notes: Values marked with different letters simultaneously show significant (P<0.05) differences.

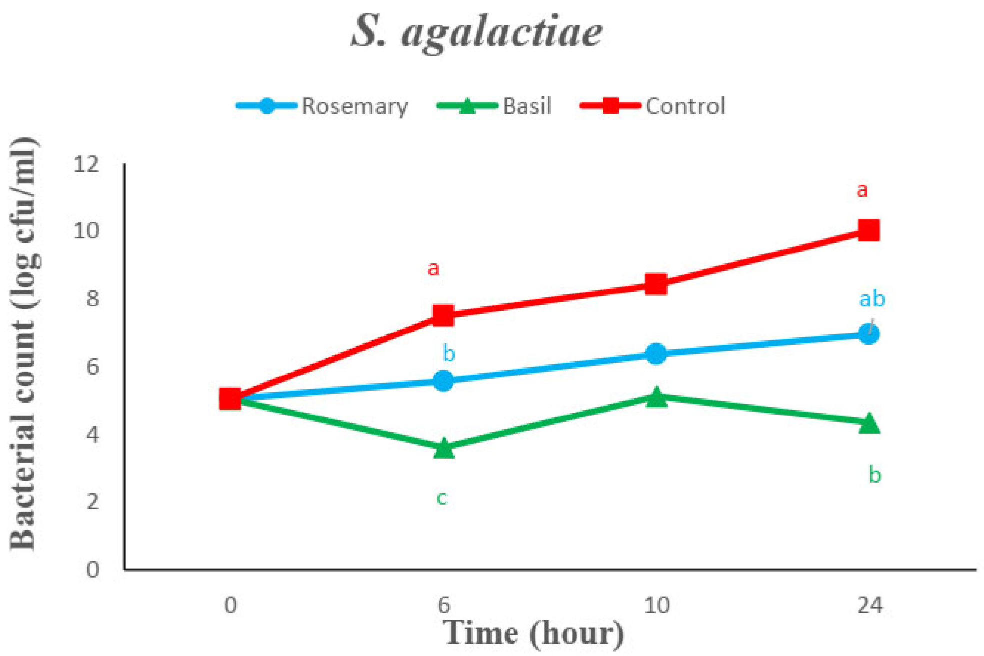

Figure 3. Growth curve of *S. agalactiae* after exposure to 0% (control, ■) and sub-mic of rosemary (●) and basil (▲) EO

Notes: Values marked with different letters simultaneously show significant (P<0.05) differences.

coli and *S. aureus* was 128 µg/mL (Rezzoug et al., 2019). In another study from Armenia, the MIC of two varieties of basil against *S. aureus* was 3.125 and 6.25 µL/mL and against *E. coli* 13 and 26 µL/mL (Avetisyan et al., 2017). In another study in Italy, the MIC of basil essential oil collected in May and October was reported to be 6 mg/mL against *E. coli* and 4 and 5 mg/mL against *S. aureus* (De Martino et al., 2021). Different studies have reported different values for MIC and MBC, which could be due to the different bacterial strains and essential oil compounds. To classify the antibacterial power of plant extracts, MIC (µg/mL) obtained by macrodilution or microdilution method is used and divided into very effective (<100 µg/mL), effective (100-500 µg/mL), mod-

Table 3. MIC and MBC of rosemary and basil EOs against bacteria compared to a positive standard antibiotic (lincospectinomycin) in milk

EOs or Antibiotic	Bacterium	MIC (%V/V)	MBC (%V/V)
Rosemary	*E. coli*	2.5	5
	S. aureus	1.25	2.5
	S. agalactiae	0.625	1.25
Basil	*Escherichia coli*	2.5	5
	S. aureus	1.25	2.5
	S. agalactiae	5	10
Lincospectinomycin	*E. coli*	2.5	5
	S. aureus	1.25	2.5
	S. agalactiae	5	10

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

erate (500-1000 µg/mL), low effect (1000-2000 µg/mL) and ineffective (more than 2000 µg/mL) (Sharifi-Rad et al., 2020). According to this classification, the EOs in the present study was effective against bacteria.

In the present study, the MIC and MBC of rosemary and basil against *E. coli* (2.5% and 5%) and *S. aureus* (1.25% and 2.5%) were the same. Although the comparison of the results of the present study in the milk media and the results of other studies in the synthetic media are not very accurate, the MIC and MBC results of rosemary and basil in the present study were higher than those of other studies on the synthetic media on rosemary (against *E. coli*: 0.3% and 0.5%; against multidrug-resistant *S. aureus*: 0.03% and 0.1% (Jiang et al., 2011), 1.5% (Esmael et al., 2020) and basil (against *E. coli*: 1.3% (Avetisyan et al., 2017), 0.6% (De Martino et al., 2021); against *S. aureus*: 0. 3% (Avetisyan et al., 2017), 0.4% (De Martino et al., 2021). According to the interaction of the fat, starch, and albumin of milk with antibacterial compounds and a decrease in the bioavailability of EOs (Burt, 2004), the above results were expected.

In the present study, the MIC and MBC of basil on three bacteria and rosemary on *S. aureus* and *E. coli* were similar to those of the lincospectinomycin antibiotic. The MIC and MBC of rosemary on *S. agalactiae* were lower than those of lincospectinomycin. These results showed the good antibacterial effect of rosemary and basil EOs.

Another noteworthy point in the present study was the stronger antibacterial effect of EOs on *S. agalactiae* and *S. aureus* (gram-positive) than on *E. coli* (gram-negative), which we expected and has been confirmed in other studies. The presence of lipopolysaccharides (LPS) (hydrophilic) in the outer membrane of gram-negative bacteria is a major obstacle for EOs, which primarily consist of hydrophobic constituents (da Silva et al., 2022). Hydrophobic properties allow relationships with bacterial membranes and mitochondria, disrupting cell structure and leading to cell death by molecules and ion leakage from the cell (da Silva et al., 2022).

Regarding the growth curve, at 6-h and 24-h the EOs had some antibacterial effects against bacterial populations that were higher against gram-positive than gram-negative bacteria. Using sub-MIC concentrations of EOs can lead to limited impact against gram-negative bacteria due to the LPS barrier hindering penetration; if the inhibitory concentration or multiple inhibitory concentrations were used, the antibacterial effects would be much stronger.

The antibacterial effects of EOs are mostly attributed to their main components (Burt, 2004). Attributing essential oil activity to a single component is similar to attributing the success of a play to a single actor. True magic lies in the interplay of major and minor components and their synergistic and antagonistic interactions (Bajalan et al., 2017).

Conclusion

The MIC and MBC results of rosemary and basil EOs in the present study in milk were higher than the expected results of other studies in synthetic media, that were expected and most results were similar to those of lincospectinomycin. The antibacterial effect of EOs is acceptable, and clinical studies are recommended to treat other diseases, including mastitis.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Study design: Reza Rahchamani, and Matia Sadat Borhani; Experiments: Saman Zarooni; Data analysis: Reza Rahchamani, and Saman Zarooni; Writing: Reza Rahchamani.

Conflict of interest

The authors declared no conflict of interest.

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