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Comparison of antibacterial activity of essential oils of *Foeniculum vulgare* Mill, *Mentha arvensis* and *Mentha piperita* against *Streptococcus mutans*

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ABSTRACT

Background and aims: Tooth decay is one of the most common chronic diseases around the world and this problem is the result of variety of different bacteria. *Streptococcus mutants* is one of the most important bacteria which is related to this disease. Finding new effective antibacterial agents is an important area in bioscience for fighting and controlling bacterial infections. Essential oils are most important natural sources of antibacterial agents, particularly against drug-resistant bacteria.

Methods: The aim of this study was to evaluate and compare the antibacterial activity of three essential *oils Mentha arvensis, Mentha piperita and Foeniculum vulgare* Mill against *Streptococcus mutants*. Disk diffusion method was carried out and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured.

Results: The results showed that all three essential oils have antibacterial activity against *S. mutants*. With a constant concentration of 100 µg/µl, the efficiency of *Mentha piperita and* then *Foeniculum vulgare* Mill was higher than the efficiency of *Mentha arvensis* at all 3 given time points (24, 48 and 72 hours). The most effective MIC and MBC were related to *Streptococcus mutants* using *Foeniculum vulgare* essential oil which were equal to 8.4 and 14.9 µg/ml, respectively. MIC and MBC for *Mentha piperita* essential oil were measured 10.5 and 16.3 µg/ml, respectively.

Conclusion: The Essential oils used in present study with different components showed antibacterial activity and therefore they can be used as new antibacterial substances.

Keywords: Chronic disease, Infection, Essential oil, Disk diffusion.

Original article

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INTRODUCTION

Tooth decay is one of the common infectious diseases in childhood.[1] Studies show different factors in prediction of this kind of disease such as microbial flora of the mouth and personal health care behaviors.[2-5] Tooth decay, in which mineral tissues of teeth are spoiled, is a kind of infectious-microbial disease.[6] However, todays it is proved that one of the most common reasons behind this kind of disease is some species of *Streptococcus mutans* which is a gram positive and is facultative anaerobe cocci in oral cavity flora. This bacteria is one of the main microorganisms involved in tooth decay procedure.

25 species of oral *Streptococcus* have been discovered until now.[7,8] Every of the species occupies a special point in the mouth. The lack of balance between oral mico floras causes oral and dental diseases. *Streptococcus mutans*, with fermentation of sucrose and producing lactic acid, can damage tooth enamel. There has been no certain program for treatment of tooth decay and dentistry costs of USA have been estimated 70.3 billion dollars.[9-11] There is a great history of the use of medicinal plants for treatment of human disease. Africa, Asia and Latin America are the most parts of the worlds which use medicinal plants.[12] Plants are used in treatment of various human diseases. For example, it is reported that essential oil of plants like Eucalyptus globulus has positive effect on treatment of Diabetes mellitus.[13] One of the most important drugs used in history of medicine which is still being used are antibiotics. Antibiotics are the main basis for microbial infection therapy. They are classified in different types according to their properties. Antibiotics, beside their use as inhibitor for bacterial growth, have some side effects on human body.

Finding new alternatives for current antibiotics is a very interesting and useful field which attracts many scientists' attentions. Moreover, the emergence of resistant bacteria to the current antibiotics is another issue that indicates the importance of finding new antibiotics. Overuse of antibiotics has become the major cause of multidrug resistance strains of several groups of bacteria.[14] Because of special characteristics of herbal extracts like flavoring effects and some of their antioxidant consequences, the use of herbs for protecting foods against microbial contaminations is increasing considerably.[15] *Mentha piperita*, is an important medicinal plant from the family of Lamiaceae and is known as *Mentha piperita*.[16]

Mentha piperita oil and its constituents are commercially used in food, pharmaceutical and cosmetics industries.[17] Menthol is used as a raw material in toothpaste, toothpowder, perfumes and tobacco. Medicinal uses of *Mentha piperita extract* are well documented. It is commonly used in soothe symptoms of the common cold and the flu. There are also some reports of the uses of *Mentha piperita* extract for relief of pain from menstrual cramp and tension headaches. *Mentha arvensis* extract has been used as antispasmodic, antiseptic and also in treating cancer in Eastern and Western traditional medicine.[18]

Mentola and *Mentha piperita* oils have moderate antibacterial effects against both gram negative and positive bacteria.[19] Singh et al indicated that *Mentha piperita* extract had ahigher antibacterial activity than ethanol.[20] *Foeniculum vulgare* is a medicinal plant from the family of Apiaceae. Its oil is mainly used as a flavoring agent in food products like bread, pickles and cheese.[21] Also, it has hepatoprotective anti-inflammatory and antioxidant effects.[22] Gulfraz et al studied antibacterial activity of *Foeniculum vulgare* Mill essential oil using disk diffusion method.[23] They reported that the essential

oil extract has significant inhibitory effect against *Bacillus cereus*, *Bacillus magaterium*, *Bacillus pumilus*, *Bacillus substilis*, *Escherichia coli* and *Candida albicans*. *Lactobacillus* is a rod-shaped Gram positive bacterium. They are usually benign, expect in the mouth where they have been associated with cavities and tooth decay. By production of lactic acid, they make environment acidic and by this way, they inhibit growth of some other bacteria.[24] It is demonstrated that streptococci family bacteria is the main cause of tooth decay, but other varieties of bacteria can cause dental caries.

To illustrate, even some lactobacillus species, beside their benefits, have been associated with dental caries. This research, therefore, is conducted to investigate the antibacterial properties of three essential oil extracts of *Mentha arvensis*, *Mentha piperita* and *Foeniculum vulgare* Mill against *Streptococcus mutants using* disk diffusion method.

METHODS

In this study, the fresh aerial parts of the herbs were used. All herbs were collected from Chaharmahal province and dried at room temperature for 3 days. The dried herb samples (500 g) were ground and subjected to hydro distillation using a Clevenger-type apparatus. The oils were dried over anhydrous Na_2SO_4 and stored at 4 °C in a sealed amber vials until the time of use.

The volatile compounds in essential oils were analyzed using GC/MS. Analytical GC were performed by Agilent 7890 A with type columns 5% HP-5 MS (column length: 30 m, internal diameter column: 0.25 µm, outer diameter column: 0.25 mm). Helium gas (whit purity 99.999%) was flowing rapidly 0.8 ml/min. The initial temperature of the column was 280 °C and the final temperature of column was 60 °C. The temperature was programmed to increase 4 °C/min. The separation ratio was set to 40:1. The injector temperature was 300 °C. In order to inject the samples, using a Hamilton syringe, 0.1 ml of essential oil was used. Analytical GC/MS was performed by Agilent 5975 C. Ionization energy in the mass spectrometer was70eV.Mass spectrum was from 50 to 550 m/z. Inhibition index (IR), were calculated for all components using homologous series of n-alkanes (C5-C25) which were injected in the same conditions as the samples. Identification of essential oil components was performed by comparing their retention times with the retention times of authentic standards and also with comparison to mass spectral analysis patterns.

The bacterial strain *S. mutans* (PTCC 1683) was prepared from Pasteur institute of Iran. Tripticase Soy Broth (TSB) and Blood Agar (BA) were used as culture medium.

Disk diffusion method was used for studying antimicrobial effects of essential oils. After 18 h of culture, liquid containing bacteria, with standard density (1×106 CFU ml-1) of 0.5 Mac Farland in Tripticase Soy Broth (TSB), was prepared and by using Sampler, 500µl of the liquid was transferred to Blood Agar (BA). The liquid was gently distributed on the surface of BA using sterile loop. There have been blank disks with 6 mm in diameter containing 30µl with concentrations 3.125, 6.25, 12.5, 25, 50 and 100 µg/µl on BA. Tested concentration of essential oils were firstly prepared and then the disks were sunk into 30µl of each concentration and dried.

Disk containing 30 µl of Water was used as negative control. The diameter of inhibition of zone was measured after 24 h of incubation at 37 °C at 24, 48 and 72 h in triplicate.

Firstly in order to determine the minimum inhibitory concentration (MIC), the suspensions of bacterial strains were

prepared from liquid cultures with standard darkness of 0.5 Mac Farland. The essential oils which were diluted 10% with water with primary concentration of 100 µg/µl and different dilutions (6 dilutions) were added to the pipes containing 10 ml of liquid culture medium. MIC of essential oils was performed using Micro well method against bacterial strain. In this step, in order to determine MIC, the 96-well plate was used which every well was added 95 µl of Tripticase Soy Broth (TSB) and 5 µl of microbial suspension. 100µl of the essential oil with concentration of 100 µg/µl was added to the first well. Then, 100 µl was taken from the first well and it was transferred to the next well. This process went on to the 6the well. The last well was contained 195µl of TSB culture medium and also 5µl of microbial suspension without any essential oil. This well was considered as negative control. In the next step, the ingredients of every well were mixed using Rotary Shaker for 20 min. Then, it was put in incubator for 24 h in an appropriate temperature (37 °C). The microbial growth was measured at 600 nm. In this study, the effect of each essential oils were determined on *S. mutans* separately with 3 replicates.

The resultant clear zones around the discs were measured in mm. The antibacterial activity of plant essential oils were indicated by clear zones of growth inhibition. Three replicates were maintained for each treatment. The data were subjected to statistical analysis by One Way ANOVA method using SPSS software and the means were compared using the Tukey method.

RESULTS

Table 1 shows the effect of different concentrations of *F. vulgare* Mill, *M. arvensis*, *M. piperita* and the control on bacteria *S. mutans* after 24, 48 and 72 hours. The results indicate that a concentration of 100µg/µl is more efficient than lower ones in all 3 substances ($P<0.05$).

Table 1: Anti-bacterial activity of different concentrations of three essential oils against *Streptococcus mutans* using disk diffusion method (zone of inhibition in mm)

Oils	***Foeniculum vulgare* Mill**			***Mentha arvensis***			***Mentha piperita***		
Concentration	**Mean ± SE**			**Mean ± SE**			**Mean ± SE**		
(µg/disk)	**24**	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**
3.12	6.41±0.0a	6.41±0.0a	6.42±0.0a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a
6.25	6.42±0.0a	6.42±0.0a	6.43±0.0a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a
12.5	6.60±0.0a	6.65±0.0a	6.65±0.0a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0 a	6.40±0.0a
25	6.76±0.0a	6.79±0.0ab	6.78±0.0a	6.48±0.0 a	6.49±0.0 a	6.49±0.0 a	6.40±0.0 a	6.42±0.0 a	6.45±0.0 a
50	7.41±0.0a	8.08±0.1b	8.21±0.0b	6.93±0.4ab	7.02±0.4ab	7.02±0.4a	7.10±0.1 a	7.23±0.1 a	7.31±0.0 a
100	9.82±0.9b	10.52±0.8c	11.06±0.7c	8.77±1.2b	8.99±1.2b	9.24±0.9b	10.48±2.0 b	10.57±1.8 b	11.25±1.9 b

Different letters on every column represent meaningful difference ($P<0.05$).

Table 1, Anti-bacterial activity of different concentrations of three essential oils against *S. mutans* using disk diffusion method (zone of inhibition in mm).

Figure 1 shows the IZ value for *F. vulgare Mill*, *M. arvensis*, *M. piperita* and negative control after 24, 48 and 72 hours while keeping a constant concentration of 100 µg/µl. Figure 1 is important from two perspectives. First, with a constant concentration of 100 µg/µl, there is a significant difference in the *means of F. vulgare* Mill, and *M. piperita* and negative control after 24, 48 and 72 hours, indicating the desirable efficiency of all 3 substances at a concentration of 100 µg/µl.

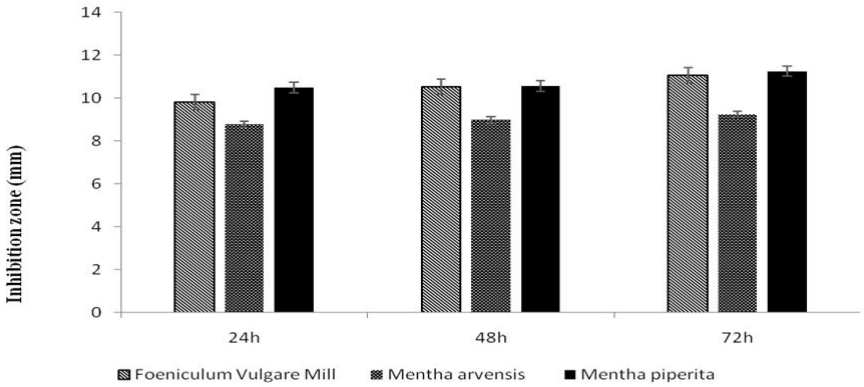

Figure 1: IZ value for *F. vulgare Mill, M. arvensis, M. piperita* and negative control after 24, 48 and 72 hours at concentration of 100 µg/ml

Furthermore, with a constant concentration of 100 µg/µl, the efficiency of *M. piperita* and then *F. vulgare Mill* was higher than the efficiency of *M. arvensis at* all the 3 given points in time, i.e. after 24, 48 and 72 hours. Figure 2 shows comparison of the changes in concentration of *F. vulgare Mill, M. arvensis, M. piperita* and negative control at a constant time.

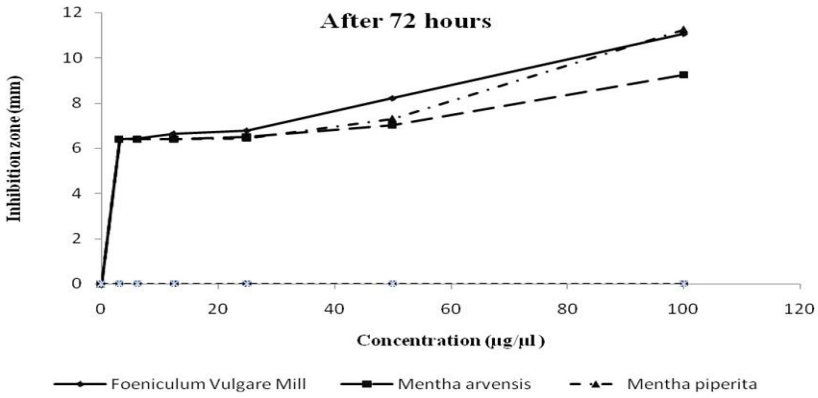

Figure 2: Changes in concentration of *F. vulgare Mill, M. arvensis, M. piperita* and negative control at a constant time

MIC and MBC of *Mentha arvensis* on *Streptococcus mutans* were measured 11.2 and 16.8 µg/ml, respectively. For *Foeniculum vulgare* essential oil it was measured as 8.4 and 14.9µg/ml, respectively which is also considered as the most effective one. For

Mentha piperita, MIC and MBC were measured 10.5 and 16.3, respectively.

The results of analyzing essential oils are summarized in Tables 2,3 and 4. According to this results Menthol (39.21%), Menthone (20.3%), Isomenthone (15.73%) and Menthyl acetate (7.02%) are four main compounds of *M. piperita* essential oil. it is showed by Pattanaik et al that menthol and Cineole have good antibacterial activity but, the whole *M. piperita* extract was more effective than its main compound.[26] In the study of antibacterial effect of *M. piperita* extract against *Klebsiella pneumoniae* it was showed that *M. piperita* extract is only effective in presence of menthol and chloroform.[27]

Table 2, Main compounds of *M. piperita* essential oil obtained by GC/mass method.

Table 2: Compositions of *Mentha piperita*

No	Compositions	%	RI
1	α-Pinene	1.08	934
2	Sabinene	0.26	973
3	β-Pinene	0.74	977
4	Myrcene	0.21	991
5	p-Cymene	0.11	1024
6	Limonene	3	1028
7	1,8-CINEOL	0.19	1032
8	α-Terpinolene	0.08	1086
9	Linalool	0.24	1099
10	Menthone	20.3	1157
11	Isomenthone	15.73	1161
12	Menthol	39.21	1180
13	Neo-Iso Menthol	1.09	1184
14	α-Terpineol	0.98	1190
15	Pulegone	1.58	1235
16	Piperitone	1.2	1250
17	Menthyl acetate	7.02	1294
18	β-Bourbonene	0.32	1378
19	β-Caryophyllene	0.76	1413
20	Germacrene-D	0.11	1475
21	Caryophyllene oxide	0.09	1588
Total			94.3

In the case of *M. arvensis essential* oil there were some similarity between compounds but in the case of major compounds no significant similarity was observable. Carvone (46.99%), 1,8 Cineole (13.53%), Borneol (8.12%) and Limonene (5.66%) were four main compounds of *M. arvensis essential* oil. Carvone is a member of terpenoids family and proposed for use as a Mosquito repellent. Ben Arfa et al studied the antibacterial activity of Crvacrol and showed that it has high antibacterial activity with significant log P of 3.52.28 *M. arvensis* essential oil contain about 0.5% Carvacrol. Antibacterial activity of Cineol specially against gram positive bacteria was showed by Bosnic et al but also it mentioned that inhibitory activity of essential oils were more than pure Cineole.[29]

Table 3, Main compounds of *M. arvensis* essential oil obtained by GC/mass method.

Table 3: Compositions of *Mentha arvensis*

No	Compositions	%	RI
1	α-Thujene	0.03	927
2	α-Pinene	1.09	934
3	Camphene	0.56	950
4	Sabinene	0.74	963
5	β-Pinene	1.59	967
6	β-Myrcene	0.41	991
7	3-Octanol	0.21	996
8	α-Terpinene	0.12	1016
9	p-Cymene	0.83	1024
10	Limonene	5.66	1028
11	1,8-Cineole	13.53	1032
12	gamma-Terpinene	0.58	1056
13	trans-Sabinene hydrate	0.06	1065
14	α-Terpinolene	0.1	1086
15	Linalool	0.37	1095
16	Menthone	0.26	1157
17	Borneol	8.12	1162
18	Terpinen-4-ol	0.48	1172
19	α-Terpineol	3.25	1185
20	Dihydrocarvone	0.69	1193
21	trans-(+)-Carveol	0.48	1217
22	Pulegone	3.28	1235
23	(-)-Carvone	46.99	1240
24	Thymol	0.67	1284
25	Carvacrol	0.47	1298
26	β-Bourbonene	0.16	1378
27	β-Caryophyllene	0.93	1413
28	α-Humulene	0.16	1446
29	Germacrene-D	0.09	1475
30	Caryophyllene oxide	0.16	1583
Total			92.07

Anethole (68.62%) and Fenchone (12.08%) are the main compounds of *F. Vulgare* essential oil. Antholes bactericidal properties were investigated and it was showed that this compound is more effective against gram positive bacteria.[30]

Table 4, Main compounds of *F. vulgare essential* oil obtained by GC/mass method. Table 5, in vitro antibacterial activity (MIC and MBC) of three essential oils against S. mutants.

Table 4: Compositions of *Foeniculum vulgare* Mill

No	Compositions	%	RI
1	α-Pinene	0.94	937
2	Camphene	0.15	951
3	Sabinene	0.33	974
4	β-Pinene	0.09	979
5	β-Myrcene	0.53	993
6	α-Phellandrene	0.23	1007
7	α-Terpinene	0.14	1016
8	p-Cymene	0.28	1025
9	Limonene	6.3	1032
10	β-Ocimene Z	0.91	1038
11	gamma-Terpinene	1.35	1057
12	Fenchone	12.08	1089
13	Camphor	0.27	1143
14	Anisole, p-allyl or Methyl chavicol	3.76	1200
15	Fenchyl acetate	0.15	1216
16	Anethole	68.62	1251
17	Thymol	1.81	1285
Total			94.3

Table 5: In vitro antibacterial activity (MIC and MBC) of three essential oils against *S. mutants*

NO	Essential oils	MIC (µg/ml)	MBC (µg/ml)
1	*Mentha arvensis*	11.2	16.8
2	*Foeniculum vulgare*	8.4	14.9
3	*Mentha piperita*	10.5	16.3

DISCUSSION

In recent years, drug resistance caused some undesirable side effects which is due to the application of chemical antibacterial materials, has encouraged the researchers to identify some new sources of herbal plants.[31] Many researchers have emphasized that the antibacterial properties of main parts of essential oils is related to their hydrophobic properties and plasma membrane of microbe. Increasing of some special ions on or inside the plasma membrane has a significant effect on motive force of protons, rate of intercellular ATP and overall activities microbial cells. Generally, the essential oils do not have same mechanism and they may prohibit bacterial growth with different methods. Most investigation about the mechanisms of essential oils has led to their effects on cellular membrane. In fact, the compositions in essential oils attack the cytoplasm membrane leading to destruction in membrane permeability, electron transport function, nutrient absorption, nucleic acid synthesis, and also ATPase enzyme activity.[32,33]

So, in this research, the effects of different concentrations of 3 essences *M. arvensis*, *F. vulgare* and *M. piperita* were tested on S. *mutans. M. arvensis*, due to the existence of menthol in it, possesses a heavy antimicrobial property.[34] In our study, the essence of this plant shows the MIC and MBC equal to 11.2 and 16.8 µg/ml, respectively. Dinesh and his coworkers examined the effect of M. *arvensis* L. essence on *S. mutans* in which MIC and MBC were respectively reported 7 and 9 mg/ml.[35] Comparison between our results with that of reported by Dinesh shows that the lower dosage of the essence has a better effect on *S. mutans*, and the essence of *Mentha possesses* antimicrobial property in decreasing the rate of biofilm formation.[36] Another plant is *F. vulgare* Mill which its extract was used in different

concentrations. There have been various experiments regarding the antimicrobial effects of the extract of F.*Vulgare* Mill. Aguiar and his coworkers in tested the antimicrobial effect of leaf extract of *F. vulgare* Mill on *S. mutans* in which MIC was reported 4000 µg/ml.[37] In our study, MIC and MBC of *F. vulgare* extract were 8.4 and 14.9 µg/ml, respectively. Comparison between our results with what reported by Aguiarrevealed that the extract of *F. vulgare* (in our study) had a better inhibitory effect on S. *mutans* than Aguiars' study. This can be due to existence of lower proportion of effective material, or bacterial resistance.[38]

M. piperita is another used plant in our study. The plant possesses high amounts of menthol and tannin. Results of a study by Sivropoulou and his coworkers showed that the antimicrobial property of *Mentha piperita* extract is related to existence of the components such as menthol, pulegone, isomenthone, piperitone, carvone and dehydrocarvone. Their results showed that menthol has an important role in creating antimicrobial property of the extract.[39]

Chami and his coworkers in introduced menthol and carvone as the most important components in creating antimicrobial property of *M. piperita* extract.[40] Also thymol and carvacrol are two important antimicrobial components. It is proved that the reaction of the extract components with each other plays an important role in antimicrobial property. Generally, it is assumed that the antibacterial effects of the herbal extracts are due to their hydrophobic property which is resulted in penetration of materials into the bacteria membrane phospholipids and mitochondria leading to bacteria death.[41] The antimicrobial activity of *M. piperita* extract had been recognized. So, it is used in drugs and toothpaste.[42] In a study by Mathur and his coworkers, the effect of *M. piperita* extract was examined against *Staphylococcus aureus, Streptococcus mutans, Streptococcus faecalis* and *Streptococcus pyogenes*. The MICs were reported 31.2, 16.6, 31.2 and 15.6mg/ml, respectively.[43] Also, Rasooli and coworkers tested the effects of extracts of different species of Mentha on *Streptococcus mutans* and *Streptococcus* pyogenes.[44] In our study, MIC and MBC for Mentha extract were 10.5 and 16.3 mg/ml, respectively. Comparison between our results with what reported by Rasooli revealed that the extract of *M. piperita*, with a lower concentration, has a better antimicrobial activity in our study. The difference in antimicrobial activities can be related to the percentage of effective components existed in the plants or to microbial resistance of the strains.[45,46]

CONCLUSIONS

Results showed that all three essential oils (*F. vulgare Mill, M. arvensis, M. piperita*) have anti-bacterial activity against *S. mutants*. In our study, *M. piperita* essential oil showed significant antimicrobial effect against S. mutants and compared with other two essential oils, it was more effective. The great importance of *S. mutans* in dental caries illustrates the need for strong and effective antibacterial compounds without any side effect or toxicity to human cells fight with this infection. Plant derived materials like essential oils are a big chance for human health and further studies is needed in this area.

CONFLICT OF INTEREST

Authors have declared that no conflicts of interest exist.

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