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Antimicrobial Effect of Spearmint and Dill oils on Yeast *Kluyveromyces marxianus* (*K. marxianus*) in Iranian Doogh

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ABSTRACT: Plant oils have many applications in food technology and antimicrobial property on a wide range of microorganisms including bacteria, yeasts, and molds. In this research, antimicrobial effect of spearmint and dill oil (in amounts of 0.1, 0.25, 0.5, 1 and 3%) has been studied on Yeast *Kluyveromyces marxianus* as one of the factors causing pollution and corruption in Iranian doogh helded in 4 and 15°C within 30 days. Results showed that oil of both plants spearmint and dill have Inhibitory effect on Yeast *Kluyveromyces marxianus* ($p<0.01$) so that the number of yeast in doogh containing the top (0.5%) of dill oil after helding in 4 and 15°C within 30 days decreased 3.5 and 4.4 log-periodic cycles, respectively, while such result was achieved for dooghs containing spearmint oil ,in concentration up to 1% (1 and 2%) indicating that the effect of dill oil is more that the one of spearmint. Results of means comparison showed that the effect of oils in reducing the number of yeasts by increasing of storage temperature is essential probably due to the greater mobility of phenolic compounds and terpenes as antimicrobial agents of essential oils. So, with attention to the restriction of using the essential oils as a natural antibacterial compounds, because they are nonpolar and affect on the flavors and aromas of the finished product and therefore consumer acceptance, the amounts of (0.5%) of spearmint oil and (1%) of dill oil are recommended in order to increase survival and improve the organoleptic properties of Iranian doogh.

Keywords: Natural preservatives, *Kluyveromyces marxianus*, Dill oil, Iranian doogh, Spearmint oil.

INTRODUCTION

Doogh is a dairy, fermentative, and acidic beverage, and Iran's native product produced by diluting the yogurt with the addition of water and salt or through direct fermentation of milk, and among the exciting drinks in the market has a special place because of the safety features. Now, its production is done both industrially and traditionally, and nutritional aspects of one can be improved by using probiotic bacteria and various additives such as essential oils and fibers. Increase of nutritive vitamins and metabolites, improvement of calcium absorption, and more digestibility than raw milk are the nutritional properties of doogh (Jakobsena *et al.*,1996).In the last decade along with the government's nutritional policy to replace the doogh instead of industrial carbonated drinks, its industrial production spread and have been largely welcomed by the people so that according to the published statistics by the bureau of statistics and information technology of ministry of agriculture jihad, doogh production and consumption (about 40%) has increased in recent years (Vosoogh *et al.*, 1388). Major flaws of doogh that reduce shelf life and its friendly market are the flavor and aroma change of the product by the action of microorganisms during its storage (Singh *et al.*, 2005; Tamime *et al.*, 2001). *Kluyveromyces marxianus* is homothal and hemiascomycete yeast that often found in dairy products. This yeast uses in the production of lactase enzyme in the industry, as source production of ribonucleic acid in the pharmacy, and as nutritional yeast in the feed for ruminant animals (Seyis and Aksoz, 2004). *K. marxianus* is one of the factors causing pollution and corruption in some dairy products and its related ones especially doogh drink in Iran which can cause swelling in its packaging (Viljoen *et al.*, 2003).

Plant essential oils known as natural compounds have antimicrobial activity on a wide range of generating corruption and pathogenic microorganisms. Most of these compounds have active phenolic groups in their structure, in fact they are considered due to having the large amounts of volatile aromatic compounds that some of which are important factors in causing taste in food (Dusan *et al.,* 2006).

Spearmint with the scientific name *Mentha spicata* is a plant native to Europe and southwestern Asia and aromatic plant with the local name "Sarsam" and the Persian name "desert spearmint" and "spike oregano". Spearmint oil has antimicrobial and antioxidant properties and is widely used in the food (as flavorings), pharmaceuticals (painkillers drugs and for the treatment of fever, headaches, asthma, and colds) and cosmetics industries around the world (Jamshidi *et al.,* 1389). Spearmint in almost all the world is known as a spice plant and grown (Fadaei *et al.,* 1389). The most abundant compound in the spearmint oil is R-carvone that gives a distinctive smell to it. Spearmint oil also contains significant amounts of limonene, hydrocarbon, 1-8 cineol, and menthol (Horemans *et al.,* 2000). The health benefits of spearmint that can be noted are anti-cough and nausea, anti-cramping, and help to digestion and reducing the symptoms of irritable bowel syndrome. In a study, (Tassou *et al.,* 2000) investigated the effect of peppermint in amounts of 0-1.2% on *S. aureus* and *S. enteritidis* in nutrient brothmedium. Accordingly, after the addition of essential oil to the medium, count of *S. aureus* colonies dropped about 6-7 log-periodic cycles. In another research, (Moreira *et al.,* 2005) studied the antimicrobial effect of plant essential oils such as eucalyptus, rosemary, peppermint, clove, lemon, oregano, and basil on different bacterial species of *E. coli* 0157:H. Results showed that the sensitivity of different species of E.coli than the essential oils used was almost similar. Antimicrobial effect of cloves oil of was more among all used essential oils. After cloves, eucalyptus and peppermint oils showed significant antimicrobial activity.

Another flavored herb is dill from the parsley family with a scientific name *Anethum graveolens* native to southwest Asia and the Middle East (Omidbigi, 2001). Dill's leaves and seeds are used as a spice and seasoning (Elgayyar *et al.,* 2001). Spearmint since ancient times has been used to relieve gastrointestinal problems and the pharmacological effects that can be cited are antibacterial, antispasmodic, and anti-fat effects (Akhondzadeh *et al.,* 2005). The most important effective material of dill is D-Karun oil but there are also some D-alpha Flandron and limonene in it (Delaguis *et al.,* 2002) in reported that Gram-positive bacteria against dill, coriander (*Coriangrum sativum*), and eucalyptus oils are more sensitive than Gram-negative bacteria. Many researchers have emphasized that the antibacterial activity of essential oils components depends on their hydrophobic properties and plasma membrane walls of microbes. (Boroomand *et al.,*1388) in study of antimicrobial property of dill and coriander seeds on *S. aureus, E. coli* O157:H7, and *Salmonella typhimuruim* showed that the mentioned essential oils have inhibitory effect on the growth of bacteria and the effect of coriander oil is more than dill one.

So today, due to the adverse effects of chemical preservatives, researchers paid special attention to the use of medicinal plants and study in this area in which the flavoring properties of some of these plant compounds have been welcomed by consumers and every day new information and useful results are provided to human society in this field applied in the prevention or treatment of diseases (Fazlara *et al.,* 1391). Therefore, the purpose of this research was the investigation of antimicrobial activity of peppermint and dill oils on *K. marxianus* yeast in Iranian doogh during storage time at different temperatures. In fact, using this natural antimicrobial factors in Iranian doogh, as one of the most dairy products in Iran, on the one hand can prevent the grow of a wide range of microorganisms such as mold and yeast on this product and the other cause to increase the organoleptic properties in doogh.

MATERIALS AND METHODS

A. Materials

Spearmint and dill oils were purchased from pharmaceutical company "Barij Esans", passed through the micro-filter with 4 micron pore slot width in order to sterilize, and finally stored at 4°C until the testing stage. Yeaet *Kluyveromyces marxianus* PTCC (5198) was prepared from Iranian Research Organization for Science and Technology. Doogh samples without additives and preservatives were prepared from Pakara Company of Sanandaj and stored in the refrigerator. In this study, tow Yeast Malt Agar and Yeast Malt Broth mediums prepared from Jamkala Sepid Company (synthesized by Merck Company) were used. Chemical materials including sodium hydroxide, chloridric acid, phenolphthalein, and amylic alcohol were also purchased from Merck Company of Germany.

B. Methods

Activation of lyophilized powder of yeast *Kluyveromyces marxianus*: To activate strain of *K. marxianus*, first, the head and mane were broken under sterile conditions and the contents of the tube containing medium YMB were inoculated. Then the pipes are wrapped with cotton and incubated for 48 h at 24°C until yeast grow and cause turbidity.

After the desired time were removed from the broth with the help of sterile ounce and cultured linearly on agar medium (YMA). Plates as upside down were incubated at 24°C for 48 hours until the number of colonies to be established. For preparation of microbial suspension, the yeast was inoculated into broth YMB after passaging three times and incubated at 25°C for 48 hours until finally the number of yeast in culture broth medium was 104 cfu/ml. The microbial suspension was placed at 4°C and used for the subculture every two weeks (Shafeei *et al.*, 1389).

Procedure: After addition of 1 cc of the microbial suspension to 100 ml of Iranian doogh samples, various amounts of peppermint and dill oils was immediately added separately to ones. Then, doogh samples containing inoculated yeast and different amounts of essential oil were stored for 30 days in 4 and 15°C. To evaluate the effect of different doses of peppermint and dill oils on the growth of the yeast *K. marxianus* in Iranian doogh during storage, the doogh bottles were sampled at different time intervals and after preparation of different dilutions, microbial culture was performed on agar medium by Pour plate procedure. Then, platesas upside down were incubated at 25 °C for 5 days and bacterial counts were performed.

C. Statistical Analysis

In this research, factorial experiment was used in a completely randomized design, data were analyzed statistically using SAS software, and the averages comparison was also performed with the same software applying Duncan's multiple range test in alpha level equal to 0.01. Process variables were essential oils (spearmint and dill), concentration of essential oils (0, 0.1, 0.25, 0.5, 1, and 3%), storage temperature (4 and 15 °C), and maintenance time (0, 7, 15, and 30 day) and the experiments were performed in triplicate. The measured feature was yeast count. Software Excel Microsoft 2010 was used for graph drawing.

RESULTS AND DISCUSSION

A. Consideration of the effect of dill oil on K. marxianusin Iranian doogh

Variance analysis results of the different concentrations effect of dill oil on doogh storage at 4 and 15 °C showed that the effect of essential oil concentration, temperature, and maintenance time variables, and also their interactions on reducing *K. marxianus* was meaningful ($p < 0.01$). The results showed that dill oil has inhibitory effect on yeast *K. marxianus* so that with increasing the essential oil concentration in Iranian doogh in the constant maintenance time the number of this yeast decreased significantly. According to data, the number of yeast *K. marxianus* in concentrations of 0.1 and 0.25%, and 0.5, 1, and 3% of dill oil reduced about 2 and 4.5 log-periodic cycles, respectively, with passing the storage time of doogh up to 30 days. So, 4.5 logarithmic cycles in the first day changed to 0 in the thirty day (Table 1).

Results of averages comparison at two different maintenance temperatures also showed that the different concentrations of essential oil in 15 °C have more effect on reducing yeast *K. marxianus* than the refrigerator temperature (4°C). This led to a further reduction of log-periodic cycle of yeasts so that in many cases this difference was statistically significant. Also, in the control samples over the storage time the growth of yeasts increased and then decreased but the decline was not significant. This reduction of growth is likely due to the acidification and decreasing of doogh pH and in conclusion preventing of the yeast growth (Table 1).

Since antimicrobial compounds of oils and essential oils are terpenes and phenolics in fact, it seems that their antimicrobial effect mechanism is similar to phenolic and terpenes compounds. More studies related to the mechanisms of phenolic compounds have been focused on their effect on cell membranes.

Table 1: Influence of different concentrations of dill oil on log-periodic cycleof K. marxianus in Iranian doogh during maintenance time.

	maintenance time (day)						
	0	**7**		**15**		**30**	
Essential oil concentration (%)		**4 °C**	**15 °C**	**4 °C**	**15 °C**	**4 °C**	**15 °C**
0/1	$4/47^{a}$	$5/04^{a}$	$3/7^{c}$	$4/2^{a}$	$3/4^{c}$	$3/44^{b}$	$2/9^{b}$
0/25	$4/4^{a}$	$4/50^{b}$	$4/26^{b}$	$4/08^{ab}$	$4/15^{b}$	$3/4^{b}$	$2/2^{c}$
0/5	$4/43^{a}$	$4/36^{bc}$	$4/32^{b}$	$2/55^{c}$	$1/58^{d}$	$1<^{c}$	0^{d}
1	$4/4^{a}$	$4/15^{c}$	$3/85^{c}$	$2/31^{c}$	$1/28^{ed}$	$1<^{c}$	0^{d}
3	$4/45^{a}$	$3/52^{b}$	$2/57^{d}$	$1/65^{d}$	$1<^{e}$	0^{d}	0^{d}
Control sample	$1<^{b}$	$4/86^{ab}$	$5/10^{a}$	$4/25^{a}$	$4/56^{a}$	$3/85^{a}$	$4/82^{a}$

*Similar letters in each column do not have meaningful difference statistically at the level $P < 0.05$.

Actually, phenolics in addition to damage to the cell membrane resulting in destruction of layers and its permeability have devastating impact on the layers action, such as disruption in electron transport, and protein and nucleic acid synthesis which can eventually lead to the inhibitory effect against microorganisms (Tassou *et al.,* 2000).Another process causing the inhibitory effect of antimicrobial compounds is destruction of the cell membranes and intracellular leakage used by many antimicrobial substances (Denyer and Hugo, 1991). Recent studies on yeast *Saccharomyces cerevisiae* have been showed that the inhibitory effect of some of the essential oils relates with the ability to form colonies. Difference in the sensitivity of the different yeasts to the essential oils depends on the cell growth stage in addition to the yeast type and chemical composition of essential oils. The cells are more sensitive to the antimicrobial effect of essential oils during cell division because the essential oils penetrate better and are therefore much effective in the germination stage (Bakkali *et al.,* 2005; Bruni *et al.,* 2003; Sacchtti *et al.,* 2005).

In the similar research, (Shafeei *et al.,* 1389) considered the extract of *Ziziphora tenuior* plant on yeast *Kluyveromyces marxianus.* Results indicated that this plant has significant inhibitory effect on this yeast. These researchers with identifying the compounds of the *Ziziphora tenuior* extract reported that phenolics and terpenes exciting in this is the main factor of its antibacterial property. Also (Delaguis *et al.,* 2002) reported that gram-positive bacteria against dill, coriander, and eucalyptus oils are very more sensitive than gram-negative bacteria. Many researchers have emphasized that the antibacterial properties of essential oil components depends on their hydrophobic property and plasma membrane walls of microbes (Sarabijamab *et al.,* 1387) in the investigating the effect of thyme oil on the activity of Lactobacillus acidophilus, starter bacteria of probiotic yoghurt, observed that the number of this bacteria significantly decreased after 7 days.

B. Consideration of the effect of spearmint oil on K. marxianusin Iranian doogh

Variance analysis results showed that Results of variance analysis showed that spearmint oil with concentrations of 3-0.1% and at different temperatures have meaningful effect on reducing yeast *Kluyveromyces marxianus* ($p < 0.01$). According to data, in constant concentration of spearmint oil with increasing the shelf life of dooghat both 4 and 15 °C temperature antimicrobial effect of this oil enhanced on yeast *Kluyveromyces marxianus* so that the most effect and as a result the minimum logarithmic number of this yeast was observed in thirty day and in 15°C. So, the increase of temperature also has a positive and significant impact on the effectiveness of spearmint oil and therefore its antimicrobial property (Table 2). The above results are according to observation of (Simsek *et al.,* 2007). These researchers in their studies showed that the number of *Kluyveromyces marxianus* and lactobacillus bulgaricus in airan samples (local doogh of Turkey) produced with mint, thyme, and garlic spices and the control sample during the maintenance time reduced significantly but the effect of mentioned spice on the number of starter bacteria are not meaningful in comparison to the control samples. As can be seen in (Table 2), with increase of essential oil concentration in Iranian doogh the number of yeast *Kluyveromyces marxianus* reduced significantly ($p < 0.01$) so that the number of this yeast decreased about 2.2 log-periodic cyclesin concentrations of 0.1 and 0.25%, 4 log-periodic cyclesin concentration of 0.5%, and 4.5 log-periodic cycles in concentrations of 1 and 3% and in the recent concentrations changed to zero (Table 2).

Generally, antimicrobial active compounds of essential oils are terpenes such as eugenol, thymol, and carvacrol with the phenolic nature.

Table 2: Influence of different concentrations of spearmint oil on log-periodic cycle of *K. marxianus* in Iranian doogh during maintenance time.

	maintenance time (day)						
	0	**7**		**15**		**30**	
Essential oil concentration (%)		**4 °C**	**15 °C**	**4 °C**	**15 °C**	**4 °C**	**15 °C**
0/1	$4/45^{a}$	$4/75^{b}$	$4/35^{bc}$	$4/1^{bc}$	$4/15^{b}$	$3/23^{b}$	$2/75^{b}$
0/25	$4/46^{a}$	$5/04^{a}$	$4/5^{b}$	$4/92^{a}$	$4/35^{ab}$	$2/4^{c}$	$2/15^{c}$
0/5	$4/50^{a}$	$4/27^{cd}$	$3/65^{d}$	$4/05^{c}$	$2/57^{c}$	1^{d}	$1<^{d}$
1	$4/43^{a}$	$4/35^{cd}$	$3/32^{e}$	$2/70^{d}$	$2/12^{d}$	$1<^{ed}$	0^{d}
3	$4/45^{a}$	$4/16^{d}$	$3/58^{ed}$	$2/85^{d}$	$1/95^{d}$	0^{e}	0^{d}
Control sample	$1<^{b}$	$4/86^{ab}$	$5/10^{a}$	$4/25^{b}$	$4/56^{a}$	$3/85^{a}$	$4/82^{a}$

*Similar letters in each column do not have meaningful difference statistically at the level $P < 0.05$.

Antimicrobial property of spearmint oil in broth medium and food system model were considered by (Sivropoulou *et al.,* 1996) and (Tassou *et al.,* 2000) and it was been shown that this property is because of the exciting of menthol, ketons such as pulegone, isomenthone, carvone, piperitone, and dihydrocarvone. In the similar research,(Karimi *et al.,*1386) studied the effect of spearmint, oregano, and rose on yeast debaryomyceshansenii in Iranian doogh at temperatures 4 and 15°C in a period of 28 days. Results showed that all of concentrations of spearmint and oregano oils at 25°C have a clear and meaningful effect in logarithmic reduction of the number of yeast than the temperature 4 °C. Also, these results accord with the study of (Moreira *et al*., 2005) considered essential oils effect as antimicrobial factors for reducing the food pathogens. According to this study, spearmint oil has good bactericidal property and is used as food preservatives. Also (Vosoogh *et al.,* 1386).with the consideration of the effect of spearmint oil on survival capability of probiotic bacteria showed that depending on the percent of used essential oil and type of microorganism spearmint oil can reduce the survival ability of the exciting bacteria in doogh. Vosoogh *et al* in a related study on the survival of bacteria bifidobacteriumlactis and lactobacillus acidophilus on doogh containing the extract of *Ziziphora tenuior* observed that this extract reduces the growth log-periodic cycle than the control sample.

By comparison of the effect of spearmint and dill oils on the reducing of the number of the yeasts log-periodic cycle, it can be concluded that the effect of dill oil is more than spearmint oil in increasing the survival time of Iranian doogh. This means that the dill oil in concentration of 5.0% could be reduce the number of yeast *Kluyveromyces marxianus* in the acceptable level (3 log-periodic cycles at 15°C) that this difference is very substantial and significant than the control sample ($p < 0.01$) while spearmint oil in concentration of 1 and 3% is resulted in decreasing the number of doogh yeast during the maintenance time.

It is also worth noting that there are restrictions in using of the essential oils because many foods have aqueous phase while essential oils and their compounds are hydrophobic. So when they were used in the food system have less effect than when used in vitro laboratory. On the other hand because these essential oils are nonpolar compounds, their use as preservative was restricted to the formulation of the fat food. Therefore, for these reasons higher concentrations of essential oils is necessary to inhibit the growth of pathogenic microorganisms in food products than their consumed amount in the medium.

However, the higher concentrations of essential oils in the food products causes reducing the acceptability of food in terms of taste and are the important restricting factor in using of essential oil as antimicrobial agent. So efforts for finding new ways to reduce the required consumed amount of essential oil have been done as both their antimicrobial properties are retained and the taste of food is desired, including the use of stabilizers, encapsulating essential oils, and using synergistic effects of mixture of essential oil and their compounds in inhibition of microorganisms growth.

CONCLUSION

Asking consumers to less use of synthetic preservatives has been led to the research and applying of the natural compounds with antimicrobial activity. Plant essential oils known as the natural compounds are widely used in the food products and have antimicrobial property on the broad range of microorganisms. In this study, the antimicrobial effect of spearmint and dill oils has been investigated in vitro on yeast *Kluyveromyces marxianus* as one of the factor causing pollution and corruption in some dairy products such as Iranian doogh. The results showed that a positive and significant impact of the essential oils both plants in reducing yeast *Kluyveromyces marxianus* in Iranian doogh during the storage time at 4 and 15 °C. Also, according to results the effect of essential oils in the higher temperature was better than the refrigerator temperature. The effect of dill oil in reducing log-periodic cycle of yeast during the maintenance time was better and further than spearmint oil. In fact, the plant essential oils have the antibacterial properties and are attractive due to large amounts of volatile aromatic compounds that some of them are of important factors starting the flavor in food. The results of this research can be useful in increasing of Shelf-life of foods, and controlling their corruption and prevention of food poisoning as well. Also, it is may be a step towards reducing the use of synthetic preservatives and increasing the use of natural ones. Therefore, the amounts of 0.5% of dill oil and 1% of spearmint oil are recommended in order to increase survival and improve the organoleptic properties of Iranian Doogh.

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