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Evaluating the Antimicrobial Effect of *Eucalyptus globulus* Oil on Yeast *Kluyveromyces marxianus*: A Study on Biopreservative in Heat Treated Noncarbonated Doogh

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ABSTRACT: The purpose of this study was to investigate the application of natural preservatives as an alternative to chemical preservatives in the production of Doogh. In order to carry out the work, different concentrations (0.25, 0.5, 0.75, 1, 1.5, 3%) of Eucalyptus essential oil were employed to study the growth of *Kluyveromyces marxianus* in Doogh at 4 °C and 15°C within 9 days. The essential oil of the plant was prepared by the hydro-distillation method and the chemical analysis was carried out by the application of Gas Chromatography -Mass Spectroscopy (GC/MS). Minimum Inhibitory Concentration (MIC) for Eucalyptus oil was determined through an agar diffusion method. The inhibitory effect and sensory characteristics of the oil were measured during 9 days of storage at 4 and 25°C. Chemical analysis of the oil indicated the presence of 15 compounds where Eucalyptol was the major constituent (78.68%) followed by γ - Terpinene (7.02%), α- Pinene (4.16%) and α- Terpinene (1.69%). The results showed that the number of *K. marxianus* in Doogh during storage time depended on the concentration of the used essential oil in the samples and storage temperature. It therefore might be concluded that the application of Eucalyptus essential oil to samples stored at 4°C and 25°C as a natural preservative might improve the desirable microbial, chemical and sensory properties of Doogh.

Keywords: *Doogh, Eucalyptus Essential Oil, Kluyveromyces marxianus, Natural Preservative, Sensory Characteristics.*

Introduction

It is over 4000 years that fermented milk products have been consumed all over the world and Doogh is one of the oldest and the most popular of these products. Today many existing products are the results of the simultaneous fermentation of the lactic acid bacteria. Essentially, the fermented milk products are produced as a method for preserving the nutrients. A wide range of products with different tastes, textures, consistency and functional characteristics is produced through milk fermentation with different microorganisms. From nutritional point of view, Doogh includes all the compounds existing in yoghurt. Doogh which is a mixture of yoghurt, water and salt, is an appropriate dairy drink, which can replace the soft drinks in food baskets. This fermented product is a unique source of high quality proteins, phosphorus, calcium,

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magnesium, zinc and B vitamins such as riboflavin, B_{12} and niacin (Buttriss, 1997).

Doogh is produced both in the large and small industrial units on high scale which shows its common use in Iran. In addition, high digestibility, having higher vitamins and nutritional metabolites in comparison with milk and increasing the calcium uptake have made Doogh a popular and nutritional product. Doogh consumption in each meal would decrease the number of pathogenic bacteria significantly and can prevent microbial contamination (Nikooyeh *et al.*, 2013).

Doogh can be produced and marketed either heated or unheated. Heated Doogh refers to a product which is undertaken a thermal process after preparing Doogh and before filling, in order to deactivate the starter microorganisms and remove possible secondary contaminations and to increase the duration of storage. This product is produced with or without carbonation. However due to the fact that Iranian consumers would prefer Doogh without carbonation, over 90% of Doogh in Iran is produced still (The Iranian National Standard No. 2453). The effective factors in reducing Doogh's shelf life and its friendly market include changing the taste and flavour of the product through the microorganism functions during its storage (Singh *et al.*, 2005; Tamime *et al.*, 2001).

Kluyveromyces marxianus yeast is widely found in diary industries. *K. marxianus* yeast is one of the contaminant factors and the causative agent of corruption in some nutrients such as yoghurt and its related products such as Doogh. This kind of yeast would ferment lactose and galactose, and create CO_2 which can cause the package of the product puffed. This yeast is thermophile which can have a higher temperature tolerance than other yeasts and reproduce through a multivariate germination method (Viljoen, 2003). Its other properties include higher thermal tolerance range, high volume of biomass and high speed growth. *K. marxianus* yeast has the highest growth speed among the other yeasts (Melani *et al.*, 2010).

There are different methods for storage of nutrients in this industry. Some methods to increase the duration of shelf life of nutrients are using heat and chemical preservatives and antibacterial compounds. Although using heat would make pathogens and pathogenic microorganisms destroyed, it is needed to increase the products' shelf life and destroy the resistant microorganisms and spores against high temperature. In addition to the economic costs, using high temperatures would decrease the quality of food. High temperature in Doogh would destroy vitamins, nutrients and its ideal properties, and denature the proteins. Using chemical preservatives has been restricted due to their poisoning features, changing nutritional and organoleptic properties of food. Because the popularity of Doogh in Iran, high growth of per capita consumption and its industrial production in recent years, it is important to present some solutions to maintain the quality of Doogh and increase its shelf life (Horie *et al.*, 2004). The chemical and physical properties of Doogh can be changed significantly during the time of storage because it has appropriate conditions for the growth and activity of yeasts, which makes it inappropriate for usage.

Today, herbal products (essence, extract...) have been recognized as natural compounds having antibacterial properties against a wide range of pathogenic factors. Iranian consumers like the presence of essence and extract of aromatic plants in Doogh. Therefore, we can use the natural essences and extracts of herbal materials in Doogh production arbitrarily. These natural essences and extracts have significant and appropriate properties which affect humans' body ideally (Soltani *et al.*, 2012).

Eucalyptus is a genus deriving from some trees from Myrtaceae family and includes nearly 800 species. Eucalyptus is native in Australia and grows in and all over the world including USA. Scientific name of their genre has been derived from 2 Greek words: "eu" means good and kalypto means hidden (Ogunwande *et al*., 2003). This tree was introduced to Iran more than half a century ago and it was planted in the south of Iran and today we can see Eucalyptus trees in Fars province. Eucalyptus essential oil has medicinal and antioxidant properties and shows antibacterial activity in a wide range of gram positive and gram negative bacteria such as *Staphylococcus aureus, Shigella dysenteriae, Salmonella paratyphi, Escherichia coli, Bacillus cereus* as well as *Candida albicans* fungi. Different studies show that the antibacterial activity of Eucalyptus is due to different compounds such as 1,8-Cineole, Citronellol, Citronelly and p-Cymene. Eucalyptus has antibiotic effects (antibacterial, antifungal, insecticideand anti-tooth decay). It decreases the dental plaque formation. It is healing, anticancer, anti-flammatory, expectorant, antioxidant. The findings resulted from animal studies show the anti-diabetic effects of Eucalyptus and it is applied in treatments of respiratory tract diseases including coughing, bronchitis (in the form of local or oral therapy) or for treatment of rheumatic pains (Nasab asgarian *et al*., 2012). Thus in the current research the antibacterial characteristic of the Eucalyptus essential oil against *Kluyveromyces marxianus* yeast and its impact on the shelf life of Doogh as well as Eucalyptus essential oil effect on physiochemical and organoleptic properties of Doogh have been investigated.

Materials and Methods

- *Materials*

The aerial components of *Eucalyptus globulus* plant were purchased from Green Life Company in Tehran. *Kluyveromyces marxianus* yeast (PTCC 5189) which is the starting culture was prepared by Iranian Research Organization for Science and Technology. Malt Agar (YMA) yeast and Malt Broth (YMB) yeast mediums were obtained from Merck Company. Chemical used such as Sodium hydroxide, Hydrochloric acid, Phenolphthalein, and Amyl alcohol were purchased from Merck chemical Company in Germany.

- *Isolation of E.globulus essential oil*

100 grams of dried leaves of the plant was placed into a flask and then the essential oil was extracted with a Clevenger-type apparatus applying the hydro-distillation method for 3 hours until there was no more essential oil. The extraction process implied streaming the vapour generated in a boiler through the bed where the plants were put on. Afterward, the solute was dragged and then it was condensed through contacting with a cold fluid. The essential oil was removed through phase separation, that was collected on top of the distillate. The oil was dried using anhydrous and kept in darkness in a sealed vial preserved at 4.00 ± 1.00 ˚C for GC-MS analysis and further usage (Basma *et al*., 2013).

- *Identification of chemical compounds in Eucalyptus essential oil*

The existing compounds in the essential oil was identified and isolated by gas chromatography attached to HP-6840/5973 model mass spectrometer (GC/MS) using HP-5MS column (30m × 0.23mm .film 0.32µm). The carrier gas was He and the gas speed was 1 ml/min and its ion energy was 70 electron volts. Identification of chemicals components was conducted through the comparison of mass spectrum of existing compounds with valid standards.

- - *Provision of yeast inoculums*

K. marxianus (PTCC 5189) strain was used in this study. The lyophilized yeast

strain was activated in YMB broth medium before the test and subcultured it in YMA medium at 25 ˚C for 48 hours. To prepare the inoculum, the yeast was inoculated into YMB after passaging 3 times and then incubated at 25°C for 48 hours until finally the number of yeast cells in culture broth medium was obtained equal to the turbidity of 0.5 McFarland standard (i.e., optical density = 0.12~0.15 at 530 nm, corresponding to 1.5×10^6 colony forming units CFU/ml) (Ah Lee & Chee, 2010).

- *Determination of Minimum Inhibitory Concentration (MIC), application of agar dilution method*

The antibacterial activity of *E. globulus* essential oil was studied using agar dilution method and the following modifications were conducted as recommended by CLSI (2016): adding the essential oil of Eucalyptus aseptically to sterile molten YMA medium, containing Tween 20 (Sigma 0.5%, v/v), with appropriate volume to produce the percentages of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3%(v/v). The resulting YMA solutions were immediately poured into Petri plates after vortexing. The YMA was used with 0.5% (v/v) Tween-20 but without essential oil as a positive growth control. The plates were spot inoculated with 5 µl (10^4CFU/ml) of yeast. The inoculated plates were incubated at 25°C for 48 hours. The end of the incubation period, the plates was evaluated to check the growth or the lack of growth of yeast. The MIC was determined as the lowest essential oil percentage in which there was a lack of growth of yeast. The presence of 1 or 2 colonies was disregarded. Each test was repeated at least twice.

- *Producing heat treated noncarbonated Doogh*

Yogurt and Doogh were produced based on the Malga dairy industry company procedure (Shazand, Arak), after receiving raw milk and confirming its physiochemical and microbial properties. The level of milk fat was standardized to 0.5% and then homogenization, pasteurization (72°C, 15min) and chilling up to 45°C were carried out. The starter culture (Saco 482) was then added and it was placed in an optimum temperature until clot formation and creating acidity of 136 Dornic Degree in yoghurt and pH of 3.4. The yoghurt was mixed well in a steel tank in order to break the the clot and unified. The next step, refined drinking water was added depending on the level of a dry matter of yoghurt which was without yoghurt fat; therefore, the level of dry matter of resulted Doogh was not lower than 2.3%. Generally the proportion of water to yoghurt was considered about 50:50. The product was mixed with 0.5% of refined salt. The resulted mixture underwent pasteurization process for 15 minutes at 72°C. The produced Doogh was homogenized (2.5 MPa) and pasteurized at 72°C, for 15 minutes.

In order to study the antibacterial effects of Eucalyptus essential oil in *K. marxianus* yeast on Doogh sample, 6 batches were prepared separately for storing at 4 and 15°C. First microbial suspension of *K. marxianus* yeast was added equally to each batch. Then 1/2MIC, MIC, 1/5MIC, 2MIC and 3 MIC concentrations of Eucalyptus essential oil were added to heat treated Doogh samples separately. A control sample was considered without the essential oil. The treated Dooghs were stored in polypropylene packages with the capacity of 100 ml for 9 days at 4 and 25°C. All the tests were repeated twice.

- *Microbial analysis*

Doogh bottles stored at 4 and 25 °C were sampled in days 0, 1^{st}, 3^{th}, 5^{th}, 7^{th} and 9^{th} and then they were cultivated using pour plate method after providing serial dilutions on an YMA culture. After that the plates were incubated for 5 days at 24°C. The microbial counting was conducted after the end of

incubation time and the results were expressed in the form of $\log_{10}$ CFU/ml.

- *Evaluation of the sensory properties*

For sensory evaluation concerned with colour, taste, flavour and overall acceptance, the 5-point hedonic scale was employed.

- *Statistical analysis and statistical society*

The tests were conducted 3 times in the form of factorial and as a fully random plan. The results from chemical, microbial and sensory tests were analysed to investigate the significant differences between data through one-way ANOVA using SPSS.23 software, and the Duncan multi-range test was used at a 5% probability level ($p<0.05$) to compare the average of treatments.

Results and Discussion

- *Eucalyptus Essential Oil characterisation*

Based on the results from analysis of chemical compounds existing in Eucalyptus essential oil by gas chromatography (Table 1), it was determined that the Eucalyptol was the most abundant compound in the essential oil. γ- Terpinene, α- Pinene, α- T erpinene, β- Myrcene, Sabinene and 4-Terpineol were present in respective decreasing order. Funchul was the least constituting compound of the essential oil. Fathi *et al.* (2009) conducted a study to evaluate the impacts of essential oil extraction methods on the level and the type of extracted compounds from Eucalyptus essential oil. The results showed that in steam distillation method, Eucalyptol (74.80-82.20%), α - Pinene (7.0-6.2-8.7%) and Limonene (6.20-5.70%) and in water distillation method, Eucalyptol (85-60-92.70%), α-Pinene (2.02-2.37%) and Limonene (2.04-3.10%) were present in the highest concentration. The conditions and methods of extraction were probably the reasons for differences in the values and the type of extracted compounds from Eucalyptus.

- *Minimum Inhibitory Concentration (MIC) of Eucalyptus essential oil against K. marxianus*

The results of antibacterial activity of different concentrations (V/V %) of Eucalyptus essential oil against *K. marxianus* yeast with an agar diffusion method are presented in Table 2. The Eucalyptus essential oil with the concentrations (V/V %) of 0.1 and 0.25 could not prevent this yeast from growing, however the yeast was prevented from growing with increasing the concentration of the essential oil and colonies were not obtained. Thus the MIC of Eucalyptus essential oil was obtained 0.5% (V/V) against K. *marxianus* yeast.

Table 1. Chemical constituents of Eucalyptus essential oil

Inverting times (min)	Compound	Percentage (%)
9.404	α -Pinene	4.16
10.189	Sabinene	1.35
10.318	β -Pinene	0.74
10.420	β -Myrcene	1.61
10.821	α -Flandron	0.86
11.000	α -Terpinene	1.69
11.394	Eucalyptol	78.68
11.709	γ -Terpinene	7.02
12.147	Trypinolen	0.67
12.362	Linalool	0.23
12.858	Funchul	0.06
13.213	Trans-pinocaroule	0.13
13.313	Camphor	0.30
13.737	4-Terpineol	1.07
13.960	Aphthorpinoleum	1.43

Table 2. Antimicrobial activity of different concentrations (V/V %) of *Eucalyptus* essential oil against *K. marxianus* through agar diffusion method

Microorganism	0.1 (%)	0.25 (%)	0.5 (%)	0.75 (%)	1 (%)	1.5 (%)	2 (%)	2.5 (%)	3 (%)	Negative control (Tween20)	Control of microbial growth
K. marxianus	+	+	-	-	-	-	-	-	-	+	+

+ No inhibition, - Inhibition

- *Evaluation of antimicrobial activity of Eucalyptus against K. marxianus in Doogh*

Based on Duncan's Multiple Range test (DMRT), it was determined that the number of *K. marxianus* yeast in Doogh samples containing different concentrations of Eucalyptus essential oil would significantly depend on the oil concentration, temperature and the storage time of Doogh, ($p<0.05$) (Table 3). Therefore, by increasing the percentage of Eucalyptus essential oil in Doogh formulation during a determined storage period, the number of *K. marxianus* yeast was reduced significantly ($p < 0.05$). In other words, the number of *K. marxianus* yeast in Doogh containing different concentrations; 0.25% (1/2MIC), 0.5% (MIC), 0.75% (1.5MIC), 1% (2MIC) and 1.5% (3MIC) of Eucalyptus essential oil showed about 1.5 to 5 logarithmic cycle reduction, until the 9^{th} day of storage time. This significant decrease was probably due to antibacterial compounds in Eucalyptus essential oil (Gilles *et al.*, 2010; Bachir & Benali, 2012). By increasing the concentration of Eucalyptus essential oil, *K. marxianus* yeast would be prevented from growing and its population in Doogh samples will be reduced as compared to the control. The main compound of Eucalyptus essential oil is monotrepenes (Maciel *et al.*, 2010). Terpenes are able to affect the cell membrane and penetrate into the lipid structure of the cell walls of microorganisms which would lead to denaturation of proteins, disrupting cellular structure, secretion of cytoplasm contents and finally the death of the cell (Oussalah *et al.*, 2005). The antibacterial effects of the Eucalyptus essential oil on *K. marxianus* yeast can be derived from the fact that this essential oil would destroy the cellular integrity and has a preventive effect on respiratory activity of mitochondria (Teixeira *et al.*, 2013; Burt *et al.*, 2004). Moreover, the growth of yeast first was increased and then decreased in the control sample during the storage time but this was not significant. This temporary drop in the number of yeasts in the control sample was probably derived from acidity and pH reduction of Doogh which prevented the yeast from growing. The comparison of counting the number of existing yeasts in a Doogh sample stored at 4 and 25°C showed that the number of *K. marxianus* yeasts existing in a Doogh sample stored at 25°C was significantly ($p<0.05$) higher than the samples stored at 4°C. The reason might be due to the increasing temperature of storage, thermal conditions would be adequate for the activity of most microorganisms which would lead to their survival. The results of this study were consistent with other researchers' studies. Najafian *et al.* (2015) applied the mint and dill essential oils in Iranian Doogh formulation and evaluated their impacts on *K. marxianus* yeast. Their results showed that by increasing the temperature of storage from 5 to 15 °C the microbial population of yeast would significantly be increased. These researchers suggested that by increasing the concentrations of these essential oils in Doogh formulation the microbial population would be reduced significantly.

- *Effect of different concentrations (v/v %) of Eucalyptus essential oil on sensory*

properties of Doogh during storage time at 4 and 25 °C

Based on the evaluation of results concerned with the sensory properties of Doogh samples containing Eucalyptus essential oil, the sensory properties of Doogh (taste, flavour, mouth feel and overall acceptance) were significantly affected by the storage temperature and essential oil concentration (P<0.05) (Figure 1). By increasing the concentration of Eucalyptus essential oil in Doogh formulation up to 0.75% at the refrigerator temperature and 1% at 25°C the sensory properties will be improved. Also the results showed that the sensory indicators of taste, flavour, colour, mouth feel and overall acceptance in the samples stored at 4°C would obtain better scores as compared to the samples stored at25°C and both scored better as compared

Table 3. Antimicrobial effect of different concentrations (V/V%) of Eucalyptus essential oil against *K. marxianus* (log $_{10}$CFU / mL) in Doogh on the 1st, 3rd, 5th, 7th and 9th at 4 and 25 °C

Temperature	Concentration	Yeast count (log_{10} CFU/g) in Doogh					
		Day 0	Day1	Day 3	Day 5	Day 7	Day 9
4°C	Control	5.17±0.00^{aA}	4.84±0.12^{aB}	4.67±0.11^{aC}	4.36±0.06^{aD}	3.93±0.18^{aE}	3.52±0.12^{aF}
	0.25%(1/2MIC)	5.17±0.00^{aA}	4.65±0.14^{bB}	4.46±0.13^{bC}	4.09±0.13^{bD}	3.73±0.14^{bE}	3.25±0.15^{bF}
	0.5% (MIC)	5.17±0.00^{aA}	3.98±0.16^{cB}	3.65±0.18^{cC}	3.28±0.10^{cD}	2.89±0.15^{cE}	2.41±0.08^{cF}
	0.75%(1.5MIC)	5.17±0.00^{aA}	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1% (2MIC)	5.17±0.00^{aA}	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1.5% (3MIC)	5.17±0.00^{aA}	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
25°C	Control	5.17±0.00^{aA}	4.93±0.10^{aB}	4.71±0.06^{aC}	4.53±0.16^{aD}	4.28±0.10^{aE}	3.97±0.13^{aF}
	0.25%(1/2MIC)	5.17±0.00^{aA}	4.81±0.12^{bB}	4.54±0.15^{bC}	4.25±0.15^{bD}	4.01±0.12^{bE}	3.73±0.16^{bF}
	0.5% (MIC)	5.17±0.00^{aA}	4.38±0.14^{cB}	4.10±0.14^{cC}	3.84±0.13^{cD}	3.57±0.17^{cE}	3.22±0.11^{cF}
	0.75%(1.5MIC)	5.17±0.00^{aA}	3.79±0.09^{dB}	3.48±0.16^{dC}	3.14±0.11^{dD}	3.86±0.08^{dE}	2.53±0.10^{dF}
	1% (2MIC)	5.17±0.00^{aA}	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1.5% (3MIC)	5.17±0.00^{aA}	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

* The a-f letters indicate a significant difference at 95% confidence level (p <0.05) in each column, and the upper case letters A-F show a significant difference in the 95% confidence level (p <0.05) in each row.

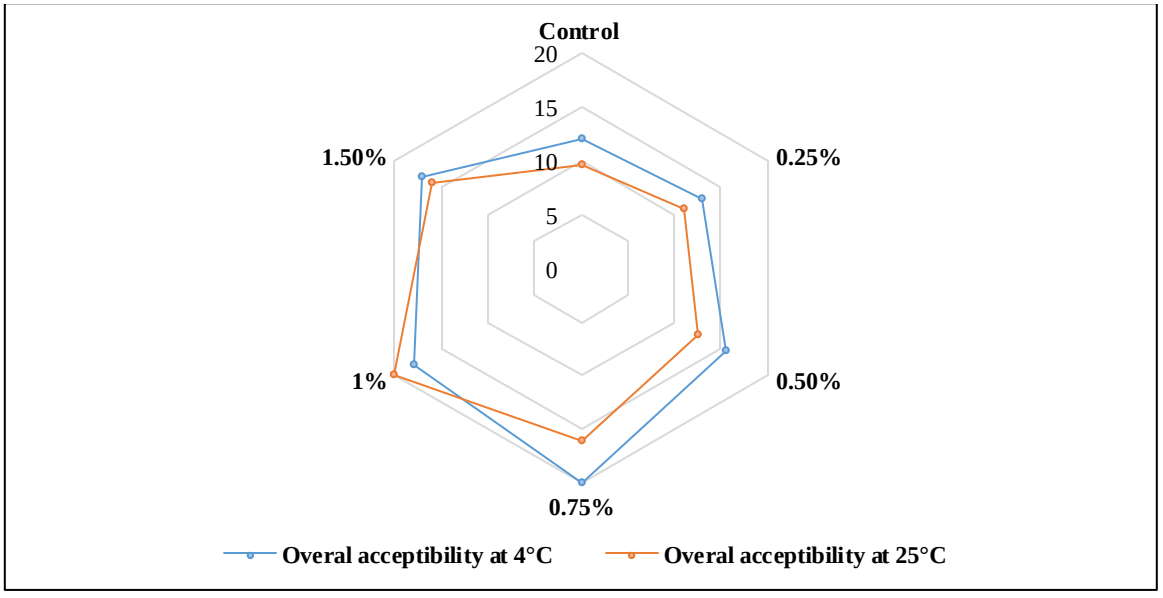

Fig. 1. Effect of Eucalyptus essential oil at different concentrations on organoleptic properties of heated Doogh on 5-point hedonic test.

to the control sample and other treatments. There might be due to the effects of Eucalyptus essential oil on reduction and prevention of microbial growth in Doogh. Undesirable characteristics will be detected if high concentrations of essential oil is applied. The results of this study were in consistent with the works carried out by other researchers.

Conclusion

The results of this study showed that the Eucalyptus essential oil has a significant and positive impact on reduction of *K. marxianus* yeast in Doogh during storage time at 4°C and 25°C. In fact, the plant essential oils are attractive due to the volatile aromatic compounds they possess, that affect the flavour of food. Therefore, this essential oil might be applied to Doogh to improve its flavour and also act as a preservative.

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